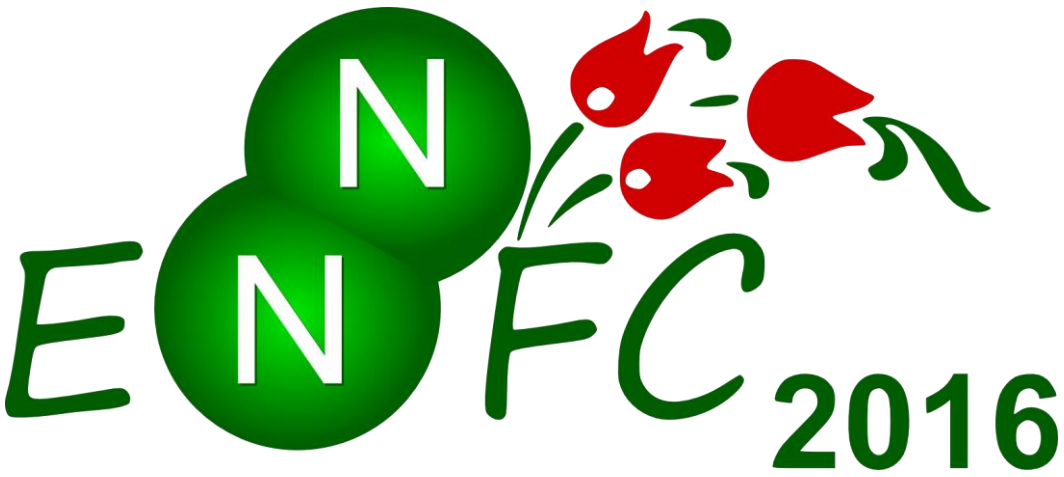
12th European Nitrogen Fixation Conference

25-28 August 2016, Budapest, Hungary
Danubius Health Spa Resort Helia ****

BOOK OF ABSTRACTS

ENNFC 2016



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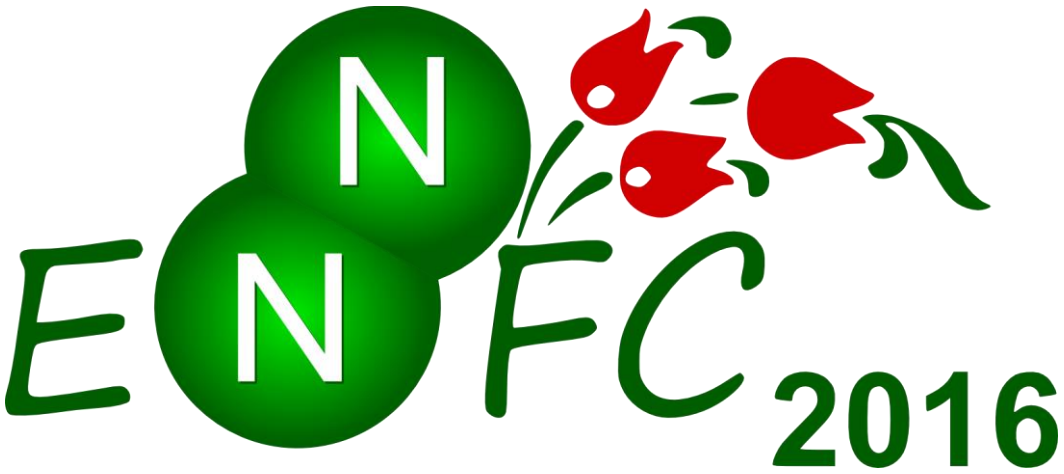


Lecture of the Kondorosi Awardee

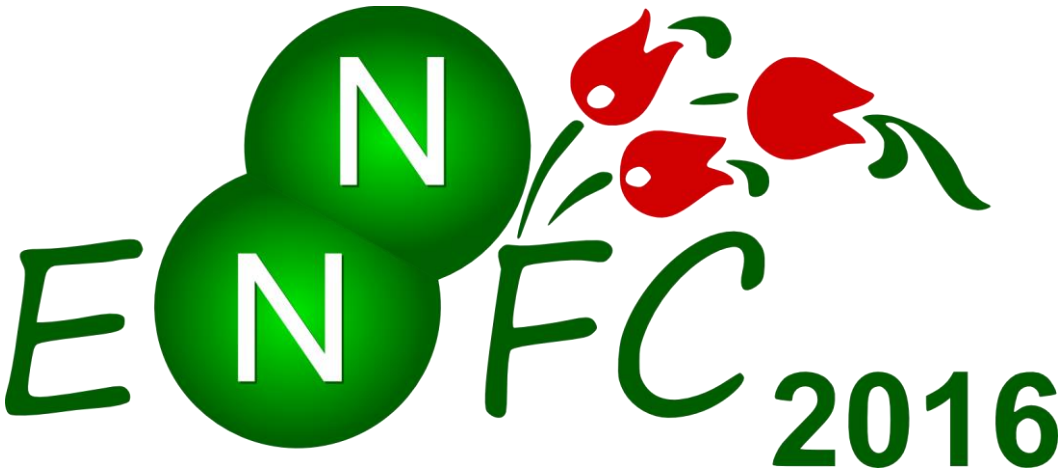
Assembly of Symbionts and Endophytes at *Lotus japonicus* Root-Soil Interface

Simona Radutoiu

Aarhus University, Denmark
radutoiu@mbg.au.dk

The association of legumes with symbiotic nitrogen-fixing bacteria is initiated by a two-way signal recognition, where an assortment of differently decorated Nod factors are produced and secreted in the rhizosphere by the microsymbiont, following perception of legume-secreted iso(flavonoids). This complex input signal is recognised at root epidermis by host receptors in a structure-dependent manner. In *Lotus japonicus*, NFR1 and NFR5 recognize the Nod factors produced by *M.loti* and initiate cellular and physiological events leading to root nodule organogenesis and infection thread formation. Nod factor recognition remains, nevertheless essential for infection thread progression towards the inner root-derived nodule primordia. Furthermore, the requirement for specific decorations is more stringent during infection, as demonstrated by legume and bacterial genetics, but the molecular mechanisms behind these different sensitivities are currently unknown. Results from the analyses of a novel host gene controlling the infection of nodule primordia in a Nod factor structure-dependent manner will be presented, and possible working models based on the new findings will be discussed.

Nitrogen fixing symbiosis in root nodules is a widespread, highly specific association identified as one of the various interactions that establish between plant hosts and surrounding microbes. To understand how the capacity of legumes to recognize Nod factors and to initiate a nodulation pathway influences their ability to interact and associate with other soil bacteria, we performed a comparative analyses of microbiota associated with soil-grown wild-type and symbiotic mutant *Lotus japonicus* plants. Community profiling of 16S rRNA gene amplicons identified a previously unsuspected role of the nodulation pathway in the establishment of distinctive bacterial assemblages in root and rhizosphere. These findings imply a role of the legume host in selecting a broad taxonomic range of root-associated bacteria that, in addition to nitrogen-fixing rhizobia, may have an impact on plant growth and ecological performance.



EMBO LECTURE

Gatekeepers of Symbiotic Nitrogen Fixation in Legumes

Jens Stougaard

Centre for Carbohydrate Recognition and Signalling, Department of Molecular Biology and Genetics, Aarhus University, Denmark

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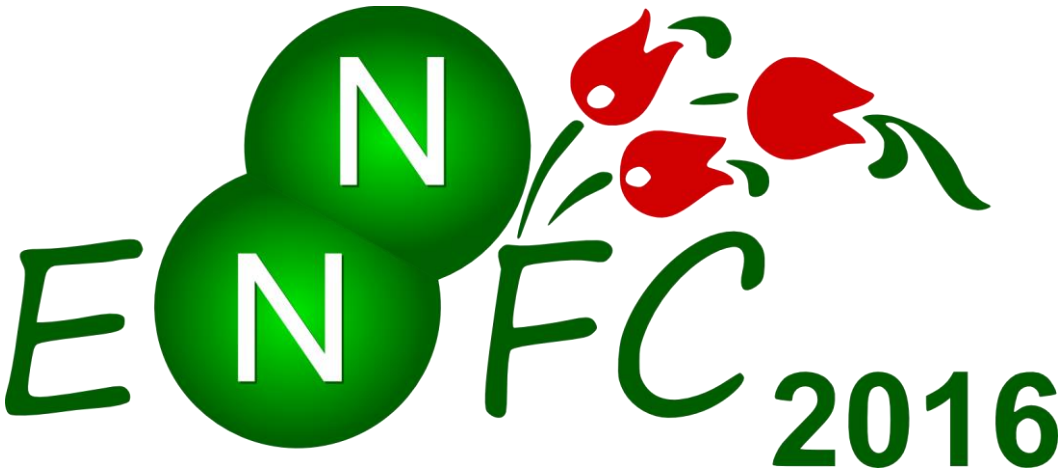
In *Lotus japonicus* a large family of LysM receptor kinases are predicted to be membrane localised and involved in perception of microbial signal molecules. A few of these receptors have been shown to play a crucial role for the interaction with rhizobia while others have so far not been studied in any detail. The function of some of these receptors in perception of signal molecules including lipochito-oligosaccharides, chitin derived signal molecules and exopolysaccharides in plant-microbe interaction will be presented. The possible role of less well described LysM type serine/threonine receptor kinases will be discussed together with the genetic and biochemical methods used for functional studies. Biochemical approaches for detailed characterization of ligand – receptor interactions in legume-rhizobial symbiosis will be presented and a model for two-step recognition of rhizobial bacteria highlighted.



PLENARY SESSION 1

Signal Perception and Transduction

Chair: Gabriella Endre

Nuclear Calcium Signalling in Symbioses

Myriam Charpentier, Jongho Sun, Teresa vaz Martins, Guru V. Radhakrishnan, Eleni Soumpourou, Richard Morris, Giles Oldroyd

John Innes Centre, Norwich, United Kingdom

Nuclear localized calcium oscillations are at the core of the common endosymbiotic pathway leading to root nodule and arbuscular mycorrhizal symbioses (1). Although these nuclear-localized calcium oscillations are essential for the activation of the endosymbiotic program, the precise mechanism that encodes the calcium signals is still poorly understood. Components involved in the generation of calcium oscillations include the potassium permeable channel, so called DMI1, and a SERCA-type calcium ATPase, so called MCA8, which are located on the nuclear membrane. The nuclear localization of those symbiotic components suggests a mechanism for the nuclear-targeted release of calcium from the nuclear envelope lumen contiguous with the endoplasmic reticulum. A mathematical modelling approach revealed that three components are essential and sufficient to recapitulate the calcium oscillation; the potassium permeable channel (DMI1), the SERCA-type calcium ATPase (MCA8) and a calcium channel (2-3). In order to identify this calcium channel, a combination of bioinformatics and reverse genetics approaches were performed. The newly identified calcium channel and its functional characterization will be presented.

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Regulatory System Evolution of Symbiotic Organ Development

Satoru Okamoto, Takema Sasaki, Emiko Yoro, Hikota Miyazawa, Takashi Soyano, Makoto Hayashi, Naoya Takeda, Yoshikatsu Matsubayashi, Hironori Fujita, Takuya Suzaki, Masayoshi Kawaguchi

Division of Symbiotic Systems, National Institute for Basic Biology, Okazaki, Japan

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The most plausible current model suggests that root nodule symbiosis might have evolved through the co-opting of genes required for arbuscular mycorrhizal symbiosis (1). This hypothesis is supported by two lines of evidence: first, the establishment of the latter type of symbiosis is estimated to be more ancient than root nodule symbiosis; and second, a number of so-called common symbiosis pathway genes have been identified that are involved in the regulation of both types of symbiosis.

On the other hand, genetic studies of auto-regulation of nodulation (AON), which regulates nodule organogenesis via long-distance signaling (2, 3), have identified a series of key genes, HAR1, KLAVIER, CLV2 and PLENTY genes in Lotus japonicus, that are orthologous or homologous to genes that play essential roles in the regulation of SAM development in non-leguminous plants (4). TRICOT, a positive regulator of nodule development, encodes a putative glutamate carboxypeptidase with orthology to Arabidopsis ALTERED MERISTEM PROGRAM 1 (AMP1) (5). Among the these genes, KLV, CLV2 and TRICOT appear to retain the ability to control SAM development while it is not known at present whether HAR1 and PLENTY are involved in regulation of the SAM. In addition, biochemical analyses indicate that arabinosylated CLE peptides have important roles in the control of nodule number (6) as well as stem cell number in SAM. Furthermore, cytokinin is known to be essential for both developmental regulations. These findings provide additional evidence for the existence of common genetic regulatory mechanisms for nodule organogenesis and SAM development.

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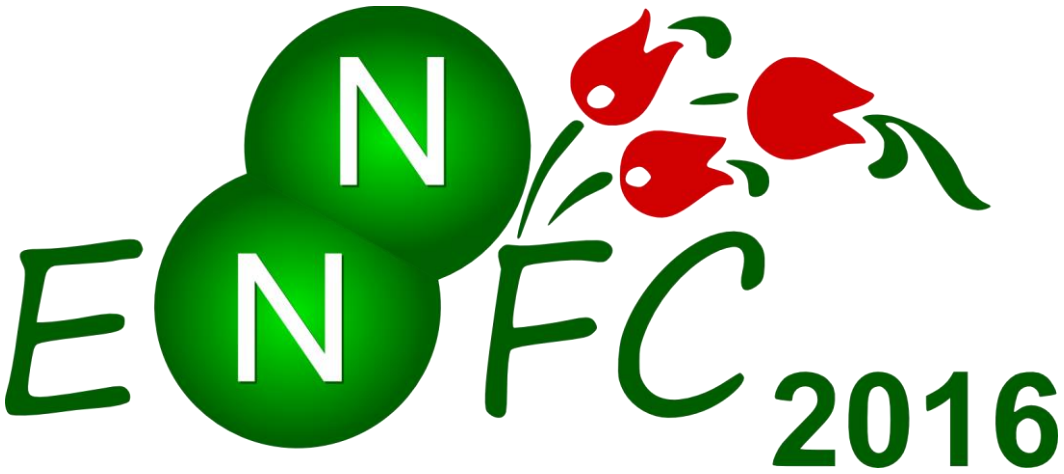

PLENARY SESSION 2

Biochemistry of Key Processes and Enzymes

Chair: Gabriella Endre

Expression and Maturation of Nitrogenase Components in Mitochondria

G. López-Torrejón[1], S. Burén[1], E. Jiménez-Vicente[1], J. A. Hernandez[2], J. M. Buesa[1], M. Veldhuizen[1], M. Navarro-Rodríguez[1], C. Echavarri-Erasun[1], Luis M. Rubio[1]

[1] *Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid, Pozuelo de Alarcón, Spain*
[2] *Department of Biochemistry, Midwestern University, Glendale, Arizona, United States*

One ambitious goal in plant biotechnology is the generation of cereals varieties that require little or no nitrogen input to achieve higher yields in a sustainable agricultural system. This goal could be achieved by engineering plants to fix their own nitrogen, i.e. by functional expression of bacterial nitrogen fixation (*nif*) genes in the plant (1,2). The most immediate obstacles this approach must overcome are the sensitivity of nitrogenase components towards O_2 and the complexity of nitrogenase, which requires a large number of genetic parts to function optimally (3).

We hypothesized that the mitochondrial matrix could provide a low O_2 environment appropriate for the assembly and the activity of nitrogenase components. To test this hypothesis *Saccharomyces cerevisiae* was chosen as model eukaryotic cell for the following reasons: (*i*) the possibility to set different O_2 levels during expression of *nif* proteins; (*ii*) its genetic amenability; and (*iii*) its well understood mitochondrially-located bacterial-like Fe-S cluster assembly machinery (4).

Here, we show that aerobically grown *S. cerevisiae* expresses active nitrogenase Fe protein when the NifH polypeptide was targeted to the mitochondrial matrix together with the NifM maturase (5). Co-expression of NifH and NifM with Nif-specific Fe-S cluster biosynthetic proteins NifU and NifS was not required for Fe protein activity, demonstrating NifH ability to incorporate endogenous mitochondrial Fe-S clusters. In contrast, expression of active Fe protein in the cytosol required both anoxic growth conditions and co-expression of NifH and NifM with NifU and NifS.

These results show the convenience of using mitochondria to host nitrogenase components, thus providing instrumental technology for the grand challenge of engineering N_2-fixing cereals.

This work was supported by Bill & Melinda Gates Foundation grants OPP1042444 and OPP1143172.

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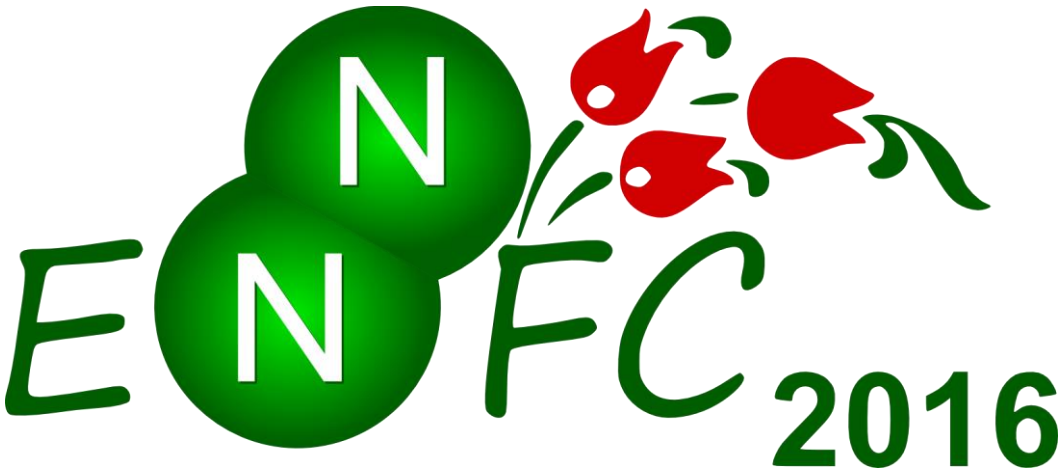

PLENARY SESSION 3

Infection and Invasion

Chair: Éva Kondorosi

Connecting the Dots, Gene Regulatory Networks in Rhizobial Infection

Cheng-Wu Liu[1], Andy Breakspear[1], Donna Cousins[1], Fran Robson[1], Kirsty Jackson[1], Da-Song Chen[2], Sonali Roy[1], Nicola Stacey[1], Christian Rogers[1], Marion R. Cerri[3] , Martin Trick[1], Giles E. Oldroyd[1], Fernanda de Carvalho-Niebel[3], Andreas Niebel[3] and Jeremy D. Murray[1]

[1] *Departments of Cell & Developmental Biology, Computational and Systems Biology, John Innes Centre, Norwich, United Kingdom*
[2] *State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Wuhan, China*
[3] *Laboratoire des Interactions Plantes Microorganismes, CNRS-INRA 2594/441, F-31320 Castanet Tolosan, France*

The formation of the infection thread (IT), a transcellular cell wall-lined compartment, serves both as a checkpoint for rhizobial recognition and as a conduit for entry of rhizobia into the developing nodule. IT formation is preceded by the establishment of a trans-cellular cytoplasmic bridge that predicts the path of the growing thread that resembles a phragmosome. We have used transcript profiling of root hairs before and during IT formation to determine the underlying processes involved. The results suggest that the formation of the infection thread involves modulation of developmental hormones including the induction of an auxin signalling module and genes involved in the cell cycle (1). To better understand the genetic switches controlling these events we profiled three infection mutants, *nin*, *nf-ya1* and *ern1*. This data revealed many potential NIN targets, including a key flavonoid biosynthetic gene. The ERN1 regulon was relatively small and did not overlap with that of NIN. Our data indicate that NIN is required for the expression of *NF-YA1* and *NF-YC2* during infection, while the induction other NF-Ys are NIN-independent, including a novel NF-Y subunit that we show is required for rhizobial infection.

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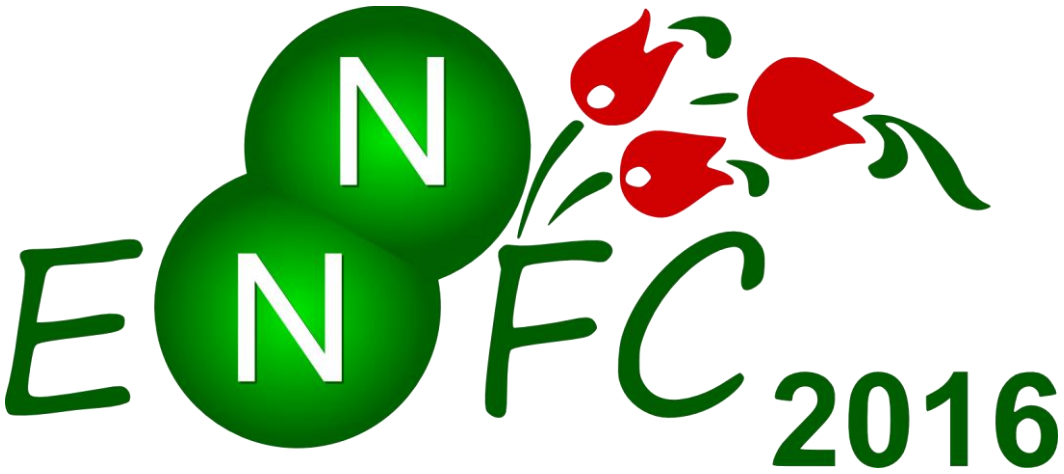
[1] *Breakspear, Liu et. al. Plant Cell. 2014 26:4680-701*

Regulation of Infection in Root Epidermis and Cortex for Nodulation

Makoto Hayashi

RIKEN Center for Sustainable Resource Science, Yokohama, Japan

Establishment of nodulation is initiated by infection of rhizobia in epidermis and cell division in cortex (CCD). Infection is typically limited in a distinct area of a root axis, so called an "infection zone", where root hairs are growing. Although almost all root hairs in the infection zone respond to rhizobia, only a few root hairs are actually infected. In addition, even if infection is successful in epidermis, the majority of them do not accompany CCD, result in failure of further infection into cortex. Meanwhile, CCD is triggered beneath the point of infection, indicating that the presence of signal(s) from epidermis to cortex. Genetically speaking, infection both in epidermis and in cortex requires a signaling pathway composed of so called common symbiosis genes. Once activated, the pathway is sufficient to trigger CCD, but infection requires additional components. In addition, the activation *per se* seems different between epidermis and cortex. Here I would like to discuss mechanistic difference of the pathway between epidermis and cortex.



PLENARY SESSION 4

Interplay of Symbiotic Interactions

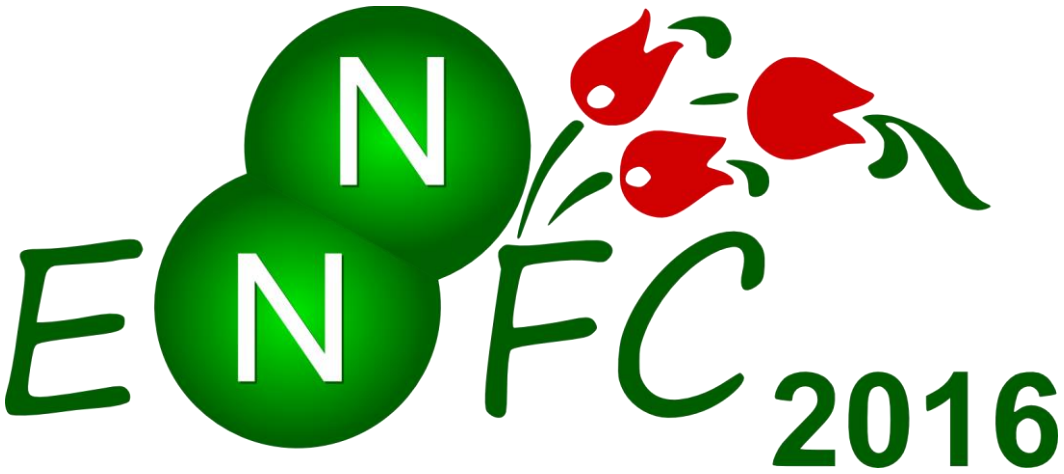
Chair: Éva Kondorosi

A Novel Component of the CCaMK/CYCLOPS Complex Regulates Root Nodule Symbiosis

Martin Parniske

University of Munich (LMU), Faculty of Biology, Genetics, Martinsried, Germany

Legumes form symbiosis with phosphate-acquiring arbuscular mycorrhiza fungi and nitrogen-fixing rhizobia. Early developmental stages of both symbioses are characterized by calcium-spiking in the nucleoplasm, which is likely to be decoded by a complex formed by a calcium- and calmodulin-dependent protein kinase (CCaMK) and CYCLOPS, a DNA-binding transcriptional activator. This complex occupies a key hierarchical position in symbiosis signaling, because deregulated versions of either CCaMK or CYCLOPS are able to induce spontaneous symbiotic root responses in the absence of microsymbionts. However, several lines of evidence indicate that additional complex components, such as DELLA, are involved in tuning the activity of the complex. Here we report the interaction with a novel complex component. We confirmed this interaction by a series of independent protein-protein and protein-DNA interaction assays. The phenotype caused by a mutant allele of the novel gene revealed its regulatory role in symbiosis. Our data suggest that the CCaMK/CYCLOPS complex is subject to dynamic changes in composition and structure, which would be in accordance with the specific spatio-temporal requirements of transcriptional regulation during the development of a symbiotic root nodule.



PARALLEL SESSION 1

Signal Perception and Transduction

Chairs: Simona Radutoiu, Pascal Gamas

DELLA-mediated Gibberellin Signaling Is a Direct Regulator of Nod Factor Signaling and Rhizobial Infection

Camille Fonouni-Farde[1], Sovanna Tan[1], Maël Baudin[2], Mathias Brault[1], Jiangqi Wen[3], Kirankumar S. Mysore[3], Andreas Niebel[2], Anouck Diet[1] and Florian Frugier[1]

[1] *Institute of Plant Sciences Paris-Saclay (IPS2), CNRS, INRA, Univ Paris Sud, Univ Evry, Univ Paris-Diderot, Université Paris-Saclay, Gif-sur-Yvette, France*
[2] *Laboratoire des Interactions Plantes-Microorganismes (LIPM), INRA, CNRS, Castanet-Tolosan, France*
[3] *Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, Oklahoma, United States*

Under nitrogen limiting conditions, legumes develop on their root system a symbiotic interaction with rhizobial bacteria leading to the formation of nitrogen-fixing nodules. Bacterial Nod Factors (NFs) are the initial trigger allowing infection in host root epidermal cells, and plant regulatory pathways modulating the NF signaling pathway are therefore critical to control nodulation efficiency. A combination of gain- and loss-of-function experiments, including mutants affecting DELLA proteins that are key negative regulators of gibberellins (GAs) signaling, revealed that this pathway inhibits rhizobial infections and controls the NF-induction of the infection marker *ENOD11* in the model legume *Medicago truncatula*. Strikingly, the ectopic expression of a constitutively dominant active DELLA protein in the epidermis is sufficient to promote *ENOD11* expression in the absence of symbiotic signals. DELLA proteins directly interact with two transcription factors, Nodulation Signaling Pathway 2 (NSP2) and Nuclear Factor YA1 (NF-YA1), which are essential for the activation of NF responses, including the expression of *ERN1* (ERF Required for Nodulation 1). In addition, DELLA proteins can interact with the *ERN1* promoter and positively trans-activate the expression of *ERN1*. Overall, these results suggest a model where the GA-dependent action of DELLA proteins may directly regulate the NSP1/NSP2 and/or NF-YA1 activation of *ERN1* transcription, subsequently regulating *ENOD11* expression and the progression of Rhizobial infections.

Identification of Genes Controlling Cytokinin Homeostasis during Nodule Development in *Lotus japonicus*

Dugald Reid[1], Ondrej Novák[2], Marcin Nadzieja[1], Anne B. Heckmann[1], Niels Sandal[1] and Jens Stougaard[1]

[1] *Centre for Carbohydrate Recognition and Signalling, Department of Molecular Biology and Genetics, Aarhus University, Denmark*
dugald@mbg.au.dk
[2] *Laboratory of Growth Regulators, Palacký University & Institute of Experimental Botany AS CR, Olomouc, Czech Republic*

Symbiotic nodule development in legumes requires the cytokinin-dependent reinitiation of cell divisions and establishment of a new root lateral organ. The mechanism by which cytokinin response is regulated in nodule establishment likely depends on spatiotemporal regulation of biosynthesis, breakdown and transport together with the availibility of receptors.

In order to determine the role of cytokinin metabolism in nodule development we identified genes in the cytokinin synthesis or breakdown pathways for further functional analysis. We recently showed that Nod Factor induces the *Lotus japonicus CYTOKININ OXIDASE/ DEHYDROGENASE3* gene, which exhibits cytokinin-degrading activity (1). At the cellular level, reporter-gene studies revealed that the *Ckx3* promoter is active during the first cortical cell divisions of the nodule primordium and in growing nodules. Cytokinin measurements and phenotypic analysis of *ckx3* mutants confirmed a role in negatively regulating root cytokinin levels in order to maintain optimal nodulation, infection thread formation and root growth.

To further clarify the role of cytokinin biosynthesis in nodule development, we also searched the *L. japonicus* genome for cytokinin biosynthesis genes and identified members of the ISOPENTENYLTRANSFERASE (IPT), LONELY GUY (LOG) and CYP735A gene families, which are expressed during nodule initiation and organogenesis. We have identified LORE1 insertion mutants or are conducting targeted knock-outs in these biosynthesis genes to further characterise their function. Mutant analysis indicated aberrant nodulation in several of these and supports a role for members of each of these families in maintaining cytokinin homeostasis during nodulation. Detailed expression analysis was also conducted using YFP fusions to the synthetic cytokinin reporter TCSn. This revealed that the TCSn promoter responds to Nod Factor dependent signalling and is active during the first cortical cell divisions of the nodule primordium and in the dividing cells of more mature nodules. Together, these findings show that cytokinin accumulation is tightly regulated during nodulation in order to balance the requirement for cell divisions with negative regulatory effects of cytokinin on infection events and root development.

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miRNAs Regulate Nodule Number in Soybean

Y. N. Wang[1], L. X. Wang[1], Y. M. Zou[2], B. J. Ferguson[3], P. M. Gresshoff[3], Xia Li[1]

[1] *College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, China*
xli@mail.hzau.edu.cn
[2] *Center for Agricultural Resources Research, Shijiazhuang, China*
[3] *Centre for Integrative Legume Research, University of Queensland, Queensland, Australia*

Organ development and organ number determination are among the greatest unsolved mysteries in plant biology. Root nodules are root lateral organs where nitrogen fixing bacteria convert atmospheric nitrogen to ammonium in legumes. It is well known that perception of Nod Factors (NF) released by rhizobia by NF receptors activates NF signaling to trigger nodule formation in legume roots, and when nodule initiation reaches certain level, a long-distance feedback mechanism, so called autoregulation of nodulation (AON) is activated to maintain the optimum nodule number. Despite the great progresses, the molecular mechanisms through which nodule development and nodule number are controlled remain largely unclear. We found that the miR172c is a key positive regulator of the NF pathway that influences nodule development in soybean. miR172c is induced by rhizobia infection and maintains at high level throughout the nodule development process. Aberrant hyperactivation of miR172c caused soybean supernodulation, whereas reduced activity of miR172c results in decreased number of nodules. We demonstrated that miR172c regulates nodulation through repressing its target gene *GmNNC1*, which encodes an AP2 transcriptional repressor of the *ENOD40* in nodulation. Through these data, we explain how miR172c and its target gene modulate nodulation in the absence and in the presence of rhizobia. Further we uncovered the GmNINa-miR172-mediated feedback loop that efficiently establish the balance of NF and AON pathways and nodule number control.

This work is mainly supported by the grants (NSFC 31230050 and 30971797) and (MOA 2014ZX0800929B and 2009ZX08009-132B).

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A Micro RNA Acts as a Signal in Systemic Control of Nodulation Symbiosis

Daniela Tsikou, Zhe Yan, Jens Stougaard and Katharina Markmann

Centre for Carbohydrate Recognition and Signalling (CARB), Department of Molecular Biology and Genetics, Aarhus University, Denmark

Legume plants such as soybean, pea and clover are globally valued for their naturally high protein content. They are important food and fodder crops that require little nitrogen fertilizer, as they cover their needs by harvesting atmospheric nitrogen through nodulation symbiosis with rhizobial bacteria.

To prevent nutritional disbalances, legumes employ a systemic feedback system tightly controlling rates of nodule development.

The phytohormone cytokinin was recently suggested to act as a shoot-to-root signal restricting symbiotic nodule emergence, thereby effecting autoregulation of nodulation (AON) in the root (1). Cytokinin controls multiple aspects of plant development, and factor(s) conveying specificity during shoot control of root symbiosis have remained enigmatic. Furthermore, the mechanisms regulating symbiotic infection ahead of nodule formation are unclear.

Here, we uncover a riboregulator, the micro RNA miR2111, that acts as a systemic shoot-to-root signal specifically regulating symbiotic infection events and nodulation by controlling mRNA levels of its target *TOO MUCH LOVE* (*TML*) in roots. In contrast to cytokinin, miR2111 acts as a positive regulator of infection that accumulates in nonsymbiotic tissues. Its levels decrease upon infection, allowing for TML to accumulate and contribute to restricting new infections as well as nodules (2). Control of both transcript levels and shoot-root travel rates of mature miR2111 depend on the receptor-like kinase HAR1, a shoot-acting negative regulator of symbiosis (3; 4) that is activated by root-derived CLE-peptides upon infection (5).

Our results open a new perspective on systemic control of nitrogen-fixing symbiosis by legume plants, pointing to a key role of a micro RNA in maintaining a susceptible default status in noninfected hosts, and ensuring a fast activation of autoregulation in the presence of compatible bacteria. AON is a central aspect of symbiotic efficiency, and understanding its mechanisms is a prerequisite for a potential transfer of nitrogen-fixing ability from legumes to other major crop plants like wheat and rice.

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POSTER 1-1 /LIGHTNING TALK/

From the Genetic Map to the Genome Assembly of the Nod Factor-independent *Aeschynomene evenia* to Shed Light on the Evolution of Nodulation

P. Leleux[1,2], C. Chaintreuil[1], L. Lamy[1,2], R. Rivallan[3], C. Klopp[2], D. J. Bertioli[4], E. Giraud[1], Jean-Francois Arrighi[1]

[1] *IRD, Laboratoire des Symbioses Tropicales et Méditerranéennes, Campus International de Baillarguet, Montpellier, France*
[2] *INRA, Plateforme GenoToul Bioinfo, INRA Auzeville, Castanet-Tolosan, France*
[3] *CIRAD, UMR AGAP, Campus de Lavalette, Montpellier, France*
[4] *University of Brasilia, Institute of Biological Sciences, Campus Darcy Ribeiro, Brasilia, Brazil*

Aeschynomene evenia has emerged as a new model legume for the deciphering of the molecular mechanisms of an alternative symbiotic process that is independent of Nod factors (1-2). Whereas most of the research on nitrogen-fixing symbiosis, legume genetics and genomics has so far focused on Galegoid and Phaseolid legumes, *A. evenia* falls in the more basal and understudied Dalbergioid clade along with peanut (*Arachis hypogaea*). In a first step to provide insights into the symbiotic genes content and the structure of the *A. evenia* genome (2n=20, 415 Mb), a genetic map was developed (3). For this, an RNAseq analysis was performed, allowing the development of molecular markers and the identification of most, but not all, symbiotic genes. Altogether, they were used to genotype a F2 mapping population, resulting in a gene-based genetic map (364 markers, 1036 cM) that was arranged in 10 linkage groups. Comparative genomic analysis with the sequenced *Arachis* genomes also indicated they are constituted of blocks of conserved macrosynteny. Thus, this genetic-map revealed the structure of the genome gene-space and, in particular, uncovered the distribution of expressed orthologs of known symbiotic genes. To further advance in our understanding of the evolution of nodulation and legume genomes, we are now engaged in an *Aeschynomene* genome sequencing project. Using the PacBio technology, a ~78x sequencing coverage of the *A. evenia* genome was obtained, resulting in a scaffold assembly and anchoring to the genetic map that represented 92% and 81% of the *A. evenia* genome size, respectively. The forthcoming completion of a reference genome sequence for *A. evenia* is anticipated to shed light on the evolution of symbiotic genes that could not be found in the *A. evenia* transcriptome datasets. It should also fasten the identification of molecular determinants of the Nod factor-independent process thanks to an ongoing mutagenesis approach by coupling gene mapping with sequencing at gene or whole genome-level.

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POSTER 1-2 /LIGHTNING TALK/

The Pea (*Pisum sativum* L.) Receptor-like Kinase Gene *LykX*, the Most Prominent Candidate for *Sym2*, is Required for Successful Penetration of Rhizobia into the Root Hair

Anton Sulima[1], Vladimir Zhukov[1], Alexey Borisov[1], Igor Tikhonovich[1,2]

[1] *All-Russia Research Institute for Agricultural Microbiology, Saint-Petersburg, Russian Federation*
[2] *Saint-Petersburg State University, Department of Genetics and Biotechnology, Saint-Petersburg, Russian Federation*

Specificity of the symbiosis between legume plants (Fabaceae) and nodule bacteria (polyphyletic group collectively known as rhizobia) is based on ligand-receptor interactions, during which the bacterial signal molecules (Nod factors) are recognized by LysM-containing plant receptor kinases (1,2). Within the pea (*Pisum sativum* L.) species, the group of genotypes originating from Middle East (so-called "Afghan peas") exists with increased selectivity towards Nod factor structure (3). This trait is controlled by plant gene *Sym2* localized in I linkage group (LG I) of pea genome and presumably encoding the Nod factor receptor (4).

Screening of pea genome BAC library (in collaboration with Dr. Helene Berges, CNRGV, France) revealed new pea gene *LykX* which is located in the *Sym2* region and encodes a receptor-like LysM kinase. We have found two specific allelic states of *LykX* resulting into varieties in amino acid composition of LysM motiff which perfectly correlate with the high or low selectivity in legume-rhizobial symbiosis. Thus, *LykX* is currently considered the most likely candidate for the *Sym2*.

For a further description of the role of *LykX* in symbiosis we ordered the TILLING of pea mutant collection (in collaboration with Dr. Marion Dalmais, INRA-URGV, France). 8 mutant families with missence mutations presumably disrupting the function of LykX protein (according to the *in silico* prediction; SIFT program) were identified. After the inoculation with *Rhizobium leguminosarum* bv. *viciae* strain RCAM1026 plants in each family have shown the decreased number of nodules along with significantly increased number of infection attempts (cases of bacterial penetration into the infection thread) in comparison to wild type plants. The earlier stages of the symbiosis appeared to be unaffected, and the nodules formed were phenotypically identical to those of wild type. To decisively confirm the role of *LykX* in selectivity towards Nod factor structure, we intend to conduct the allelism test between *lykX* mutants and "Afghan" forms of pea.

This work was supported by RSF grant 14-24-00135, RFBR grant 15-29-02737 and Grant of President NSH6759.2016.4.

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POSTER 1-3 /LIGHTNING TALK/

Lotus-Rhizobium Symbiosis is Facilitated by the Epidermal Nod Factor Receptor

Eiichi Murakami, Jeryl Cheng, Zoltán Bozsóki, Kira Gysel, Yasuyuki Kawaharada, Lene H. Madsen, Jens Stougaard & Simona Radutoiu

Department of Molecular Biology and Genetics, Aarhus University, Denmark

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Nitrogen fixing symbiosis initiates in most legumes in the epidermis where a more relaxed sensitivity for the structure of bacterial Nod factors exists, compared to the later stages of the interaction which unfold in the inner cortical layers (1). Identifying components that contribute to this differential sensitivity is essential for understanding how legume-rhizobia specificity is acquired. In *Lotus japonicus*, nanomolar concentrations of *M.loti* Nod factors are detected in the root hairs by the NFR1 and NFR5 receptor kinases that control the tightly coordinated molecular, cellular and physiological events leading to root nodule symbiosis (2, 3). NFR1 and NFR5 are the founders of LysM receptor kinase family, that in legumes has greatly expanded through whole genome or tandem duplications (4). The central role of NFR1 and NFR5 in root nodule symbiosis concealed so far, the contribution of the other LysM receptors to the symbiotic development. We have identified an additional LysM receptor that contributes to Nod factor perception and signalling in the epidermis. Our results obtained from phenotypic analyses of mutant plants coupled with tailored genetic complementation studies, and biochemical investigations indicate that complex signalling hubs are assembled at different stages of the symbiosis, where the assortment and contribution of LysM receptors is regulated transcriptionally and biochemically.

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POSTER 1-4 /LIGHTNING TALK/

Identification of a Novel Component of the CCaMK/CYCLOPS Complex

Katja Katzer and Martin Parniske

University of Munich (LMU), Faculty of Biology, Genetics, Martinsried, Germany

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Early developmental stages of the agriculturally and ecologically important plant root symbioses with phosphate-acquiring arbuscular mycorrhiza (AM) fungi and nitrogen-fixing bacteria share common signaling components. A complex of a calcium- and calmodulin-dependent kinase (CCaMK) and the transcriptional regulator CYCLOPS represents the last step of the common symbiotic pathway before the bifurcation of signal transduction, but the mechanism underlying the developmental decision process remained unknown. Several lines of evidence indicate that additional complex components are involved in tuning the activity of the complex. Here we provide the first structural insights into CYCLOPS regulation during root symbiosis. Using x-ray crystallography, part of the CYCLOPS DNA-binding domain was structurally solved. Based on the protein structure, a novel component of the CCaMK/CYCLOPS complex could be predicted and interactions confirmed by independent protein-protein and protein-DNA interaction assays. Mutational analyses to weaken or strengthen the conformational integrity, demonstrated the importance of the structure for CYCLOPS' activity and regulation.

POSTER 1-5 /LIGHTNING TALK/

KNAT3/4/5-like KNOX Transcription Factors Regulate Symbiotic Nodule Organ Development in *Medicago truncatula* Potentially Through the *MtEFD*/*MtRR4* Cytokinin-Related Regulatory Module

Carole Laffont[1], Elisabetta Di Giacomo[2], Francesca Sciarra[2], Maria Adelaide Iannelli[2], Florian Frugier[1] and Giovanna Frugis[1]

[1] *Institute of Plant Sciences Paris-Saclay (IPS2), CNRS, INRA, Université Paris Sud, Université Evry, Université Paris-Diderot, Université Paris-Saclay, Gif-sur-Yvette, France*
[2] *Istituto di Biologia e Biotecnologia Agraria (IBBA), Operative Unit of Rome, Consiglio Nazionale delle Ricerche (CNR), Monterotondo Scalo (Roma), Italy*

Class 1 KNOX homeodomain transcription factors (TFs) are involved in plant shoot development and leaf shape diversity, whereas Class 2 *KNOX* genes are less characterized, even though an antagonistic function relative to Class 1 KNOXs was recently proposed. We investigated the role of *KNOX* genes in legume root nodule organogenesis using the *Medicago truncatula* model. *In silico* expression data together with GUS transcriptional fusions identified three *Mt KNAT3/4/5-like* genes expressed during nodulation from the early primordia stages. *Mt KNAT3/4/5-like* genes encode four highly homologous proteins expressed during nodule organogenesis and in overlapping zones of the nodule, suggesting functional redundancy. A simultaneous RNAi-mediated silencing of *Mt KNAT3/4/5-like* genes provoked an increased formation of fused nodule organs, correlated with a decreased expression of two genes associated to nodule organogenesis: the *MtEFD* (*Ethylene response Factor required for nodule Differentiation*) TF (1) and its direct target *MtRR4,* a type A cytokinin Response Regulator gene expressed in nodule primordia (2). This suggests that *Mt KNAT3/4/5-like* genes therefore regulate legume nodule development potentially through the MtEFD/MtRR4 cytokinin-related regulatory module and may contribute to the diversity of nodule shapes.

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POSTER 1-6 /LIGHTNING TALK/

Nodule and Lateral Root Development Are Mediated by Independent Pathways Downstream of the MtCEP1 Peptide / CRA2 Receptor in *Medicago truncatula*

Nadiatul A. Mohd Radzman[1], Carole Laffont[2], Ariel Ivanovici[1], Neha Patel[1], Dugald Reid[3], Jens Stougaard[3], Florian Frugier[2], Nijat Imin[1] and Michael A. Djordjevic[1]

[1] *Plant Science Division, Research School of Biology, College of Medicine, Biology and the Environment, Australian National University, Canberra, Australia*
[2] *Institute of Plant Sciences Paris-Saclay (IPS2), CNRS, INRA, Univ Paris Sud, Univ Evry, Univ Paris-Diderot, Université Paris-Saclay, Gif sur Yvette, France*
[3] *Plant Molecular Biology, Department of Molecular Biology and Genetics, Aarhus, Denmark*

C-TERMINALLY ENCODED PEPTIDEs (CEPs) are important non-cell-autonomous regulators of root system architecture that are secreted to the apoplast (1-3). In *Medicago truncatula* (*Mt*), MtCEP1 is upregulated in roots by nitrogen limitation which is required for nodulation competency. *MtCEP1* over expression or addition of MtCEP1 peptide to roots increases nodule number and inhibits lateral root emergence (1, 2). MtCEP1 peptide-dependent nodulation phenotypes were found to require the symbiotic (SYM) signalling pathway and MtEIN2 (Mt ETHYLENE INSENSITIVE 2) but acted independently of SUNN. MtCEP1 inhibition of lateral root emergence acted through a separate and MtEIN2-independent mechanism. We investigated how MtCEP1 enhances nodulation. Raising MtCEP1 levels increases the number of rhizobial infections, extends the developmental competency of roots for nodulation and leads to the formation of "fused" nodules with multiple nodule meristems. Nodule formation occurs at both proto-xylem and proto-phloem poles. These results suggest that the MtCEP1 peptide decreases MtEIN2-dependent responses that negatively regulate nodule formation. Accordingly, MtCEP1 counteracts the phenotypic effects of increasing ethylene precursor concentrations and an ethylene synthesis inhibitor treatment antagonises MtCEP1 nodulation phenotypes. MtCEP1 also inhibits the development of MtEIN2-dependent pseudonodule formation. A mutant affecting the MtCRA2 (COMPACT ROOT ARCHITECTURE 2) receptor was examined due to its close homology to the *Arabidopsis* CEP Receptor 1 (4). The *cra2* mutant was found to be unresponsive to MtCEP1 effects on lateral root and nodule formation. This suggests CRA2 is a CEP peptide receptor that mediates both organogenesis programs. In addition, an ethylene inhibitor treatment complements the *cra2* nodulation, but not the lateral root phenotypes. These results indicate that MtCEP1 and its likely receptor, CRA2, act through ethylene-dependent and independent pathways to regulate important aspects of root system architecture.

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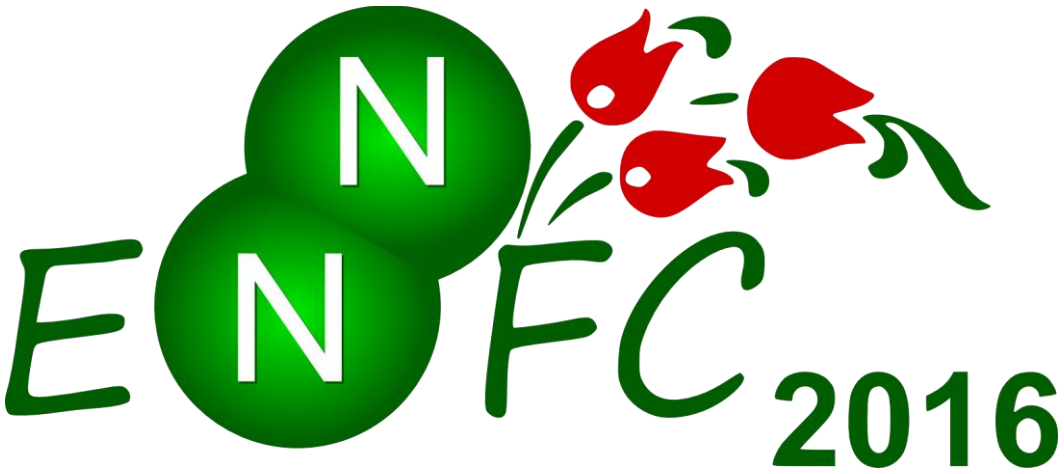

PARALLEL SESSION 2

Biochemistry of Key Processes and Enzymes

Chairs: Ray Dixon, Christian Staehelin

Genetic Requirements for Biosynthesis and Activity of FeFe Nitrogenase

Jianguo Yang[1], Xiaqing Xie[1], Xia Wang[1], Ray Dixon[2] and Yi-Ping Wang[1]

[1] *State Key Laboratory of Protein and Plant Gene Research, College of Life Sciences, Peking University, Beijing, China*
[2] *Department of Molecular Microbiology, John Innes Centre, Norwich, United Kingdom*

All diazotrophic organisms sequenced to date encode a molybdenum-dependent nitrogenase, but some also have alternative nitrogenases that are dependent on either vanadium (VFe) or iron only (FeFe) for activity. In *Azotobacter vinelandii*, expression of the three different types of nitrogenase is regulated in response to metal availability. The majority of genes required for nitrogen fixation in this organism are encoded in the nitrogen fixation (*nif*) gene clusters, whereas genes specific for vanadium- or iron-dependent diazotophy are encoded by the vanadium nitrogen fixation (*vnf*) and alternative nitrogen fixation (*anf*) genes, respectively. Due to the complexities of metal-dependent regulation and gene redundancy in *A. vinelandii*, it has been difficult to determine the precise genetic requirements for alternative nitrogen fixation. In this study, we have used *Escherichia coli* as a chassis to build an artificial iron-only (Anf) nitrogenase system composed of defined *anf* and *nif* genes. Using this system, we demonstrate that the pathway for biosynthesis of the iron-only cofactor (FeFe-co) is likely to be simpler than the pathway for biosynthesis of the molybdenum-dependent cofactor (FeMo-co) equivalent. A number of genes considered to be essential for nitrogen fixation by FeFe nitrogenase, including *nifM*, *vnfEN*, and *anfOR*, are not required for the artificial Anf system in *E. coli*. This finding has enabled us to engineer a minimal FeFe nitrogenase system comprising the structural *anfHDGK* genes and the *nifBUSV* genes required for metallocluster biosynthesis, with *nifF* and *nifJ* providing electron transport to the alternative nitrogenase. This minimal Anf system has potential implications for engineering diazotrophy in eukaryotes, particularly in compartments (e.g., organelles) where molybdenum may be limiting. Our latest progress on further simplification of the minimal Anf system to functionally substitute the electron transport chain with electron donors from the chloroplast will be discussed.

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Yang JG, Xie XQ, Wang X, Dixon R, Wang Y-P. Reconstruction and minimal gene requirements for the alternative iron-only nitrogenase in Escherichia coli. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(35):E3718-E3725. doi:10.1073/pnas.1411185111.

The Role of Rhizobial (NifV) and Plant (FEN1) Homocitrate Synthases in Symbiotic Nitrogen Fixation

Nico Nouwen, Jean-François Arrighi, Fabienne Cartieaux, Djamel Gully, Clemence Chaintreuil and Eric Giraud

IRD, Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), Montpellier, France

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For a long time it has been remarkable that many rhizobia do not contain a *nif*V gene, which encodes for a homocitrate synthase that catalyzes the condensation of acetyl coenzyme A and 2-oxoglutarate. Homocitrate is a component of the FeMoCo-factor present in the catalytic center of dinitrogenase which is absolutely required for a proper functioning of the nitrogenase enzyme complex (1, 2). Recently, it has been demonstrated that *Lotus japonicus* expresses a nodule specific homocitrate synthase (FEN1) and that the contaminant production of homocitrate compensates for the absence of homocitrate synthase activity in the bacterial partner *Mesorhizobium meliloti* (3).

Obviously, diazotrophs that fix dinitrogen in free-living conditions need a way to produce homocitrate themselves. Therefore, it is no surprise that rhizobia like *Azorhizobium caulinodans* ORS571 and photosynthetic bradyrhizobium strains that fix dinitrogen in the free-living state contain a *nif*V gene. However, if the *nif*V gene as found in these rhizobia is required for dinitrogen fixation in the symbiotic state and if the host plant always supplies the rhizobium with homocitrate is not known. To investigate this, we have constructed a Δ*nif*V mutant in the model photosynthetic *Bradyrhizobium* strain ORS285. Moreover, we have analyzed the presence and expression of *fen*1 gene homologues in several *Aeschynomene* species that establish a symbiotic interaction with the *Bradyrhizobium* ORS285 strain. Data of this study will be presented.

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The Role of the Signal Peptide Peptidase in Nodule Development and Symbiosis

Rui Lima, Attila Farkas, Attila Szűcs, Edit Ábrahám, Edina Kiss, Hilda Tiricz, Olga Nagy, Attila Kereszt, Éva Kondorosi

Institute of Biochemistry, Biological Research Centre HAS, Szeged, Hungary

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The signal peptide peptidase (SPP) is a conserved intramembrane aspartyl protease which processes signal peptides (SP) arising from cleavage of secretory preproteins in the endoplasmic reticulum (ER) by the signal peptidase (1). SPP can clean ER from the remnant signal peptides but it can also liberate small biologically active oligopeptides from them.

In *Medicago truncatula* root nodules, several hundreds of secreted nodule specific cysteine-rich (NCR) peptides are produced in the symbiotic cells (2). The signal peptides of the NCRs are relatively conserved unlike the highly diverse mature NCR peptides (3). As NCRs are produced in large amounts all along the differentiation of symbiotic cells, removal of their signal peptides from the ER might be essential for normal cell functioning. On the other hand it cannot be excluded that conservation of the NCR signal peptides is maintained for generation of SP-derived oligopeptides with biological activity. One of the two *M. truncatula* SPPs is highly nodule-specific, thus it is plausible that its function might be important in nodule development and bacteroids differentiation via processing SPs of NCRs and other nodule-specific genes.

We created several stable transgenic lines, where the nodule-specific (ns) SPP mRNA level is reduced to 3-10% of the wild type level by RNA-interference (RNAi). Phenotypic and molecular analysis of the RNAi nodules demonstrate crucial role for this SPP in nodule development. The nodules developed on these lines were small and Fix$^-$. In line with the expression pattern of nsSPP, cellular invasion was normal, but the bacteroids were only slightly elongated. NCRs necessary for further differentiation of bacteroids were down-regulated or not produced and as a consequence of this formation of the ZIII was aborted.

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Sinorhizobium meliloti YbeY: A Novel Endoribonuclease Involved in RNA-mediated Gene Silencing

José I. Jiménez-Zurdo[1], Marta Robledo[1], Alexandra Peregrina[1], Natalia I. García-Tomsig[1], Margarida Saramago[2], Rute G. Matos[2], Cecilia Arraiano[2], Rolf Hilker[3], Anke Becker[3]

[1] *Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Granada, Spain*
[2] *Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Oeiras, Portugal*
[3] *LOEWE Center for Synthetic Microbiology and Faculty of Biology, Philipps Universität, Marburg, Germany*

The *ybeY* gene integrates the proposed minimal prokaryotic genome. Structural studies of YbeY orthologs revealed a conserved three histidine H(X)3H(X)4DH motif shared by metallo-hydrolases and global similarity to the MID domain of eukaryotic AGO proteins involved in RNA-directed gene silencing. These features suggest that YbeY could serve catalytic and/or Hfq-like RNA-binding/chaperone functions in bacteria, as suggested by previous studies in *E. coli* and the legume symbiont *Sinorhizobium meliloti* (1-3). We have biochemically and genetically characterized the YbeY ortholog of *S. meliloti* (*Sm*YbeY). Co-immunoprecipitation (CoIP) with a FLAG-tagged *Sm*YbeY yielded a poor enrichment in RNA species, compared to Hfq CoIP-RNA uncovered previously by a similar experimental setup (4). Purified *Sm*YbeY behaved as a monomer that indistinctly cleaved double- and single-stranded RNA substrates, a unique ability among bacterial endoribonucleases. *Sm*YbeY-mediated catalysis was supported by the divalent metal ions Mg^{2+}, Mn^{2+} and Ca^{2+}, which influenced differentially on cleavage efficiency and reactivity patterns. Ca^{2+} specifically impaired *Sm*YbeY activity on structured RNA molecules. *Sm*YbeY loss-of-function compromised expression of housekeeping genes related to core energy and RNA metabolism, whilst promoting accumulation of motility, transport and late symbiotic transcripts. Some of the upregulated mRNAs might be *Sm*YbeY substrates which are targeted by sRNAs that bind Hfq. A genetic reporter assay confirmed that *Sm*YbeY participates in sRNA-mediated down-regulation of the amino acid ABC transporter *prbA* mRNA. We have thus discovered a bacterial endoribonuclease with unprecedented catalytic features, acting also as novel silencing enzyme in riboregulation.

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POSTER 2-1 /LIGHTNING TALK/

Comparative Biochemical Studies of *Lotus japonicus* LysM Receptor Like Kinases

Kira Gysel[1,5], M. Vinther[1,5], M. Blaise[1,2,5], J. Cheng[1,5], C. T. Hjuler[1,3,5], Y. Kawaharada[1,5], A. Muszynski[4], M. B. Thygesen[1,3,5], K. R. Andersen[1], S. S. Thirup[1,5], K. J. Jensen[1,3,5], J. Stougaard[1,5]

[1] *Department of Molecular Biology and Genetics - Aarhus University, Denmark*
[2] *Centre d'etudes d'agents Pathogénes et Biotechnologies pour la Santé (CBPS) - Université de Montpellier, France*
[3] *Department of Chemistry, University of Copenhagen, Denmark*
[4] *Complex Carbohydrate Research Center, University of Georgia, United States*
[5] *Centre for Carbohydrate Recognition and Signalling, Aarhus University, Denmark*

Two receptors in the model legume *Lotus japonicus*, NFR1 and NFR5, recognize lipochitooligosaccharides (Nod-factors) secreted by *Mesorhizobium loti*. NFR1 and NFR5 belong to a large family of LysM receptor kinases (LysM-RK) in *Lotus japonicus* containing 3 extracellular LysM domains and an intracellular kinase or pseudokinase domain connected by a short transmembrane domain. LysM-RK are key players in friend-or-foe recognition and are capable of recognizing and distinguishing structurally very similar signaling molecules and initiate appropriate local and systemic responses to those. Additionally, the LysM-RK EPR3 in *L.japonicus* was recently shown to perceive *M.loti* exopolysaccharides (EPS) and regulate bacterial entry (1) .

LysM-RK and their ligands are therefore interesting targets for comparative binding affinity studies.

However, biochemical studies of full-length LysM-RK are challenging due to the recalcitrant nature of these single-pass membrane proteins, resulting in laborious recombinant expression, difficult purification and low yields. We developed and optimized an insect cell-based system for recombinant expression of a variety of ligand-binding LysM-RK ectodomains to facilitate purification, obtain higher yields and enable advanced ligand binding assays.

Here, we present the results of binding studies on insect-cell expressed LysM-RK by Microscale thermophoresis and Biolayer interferometry. As an example, the direct binding of the EPR3 ectodomain to *M. loti* EPS, a ligand not containing N-acetyl-D-glucosamine, could be successfully demonstrated with a dissociation constant $K_D = 2.7 \pm 0.2$ μM (1) .

References:

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POSTER 2-2 /LIGHTNING TALK/

Regulation of Bacterial Metabolism by the Phosphotransferase System (PTS^{Ntr})

Carmen Sanchez-Cañizares[1], Francesco Pini[1], Jürgen Prell[2], Philip Poole[1]

[1] *Department of Plant Sciences, University of Oxford. South Parks Road, Oxford, United Kingdom*
[2] *Soil Ecology, RWTH, Aachen, Germany*

The establishment and maintenance of an effective N-fixing symbiosis is intimately interconnected with the metabolism of the plant and requires a complex coupling of biochemical and morphological factors between rhizobia and their host (1, 2). The success of this interaction relies on a fine-tuned coordination of intracellular and extracellular signals, which in the case of the bacterium seems to be exerted by the phosphoenolpyruvate: carbohydrate-phosphotransferase system (PTS). This PTS system is the key signal transduction pathway involved in the regulation of carbon metabolism in Gram-negative and Gram-positive bacteria (3). It acquires the phosphate from phosphoenolpyruvate and passes it through the different components of the system, with the ultimate acceptor being a sugar available in the environment, which is phosphorylated upon transport by the membrane components EIIB and EIIC. In the case of Gram-negative bacteria, PTS^{Ntr} represents an alternative PTS system encoded by the genes *ptsP, npr* and *ptsN*, which preserves the phosphotransfer components, but lacks the permeases, suggesting an exclusively regulatory role (4). Additionally, in *Rhizobium leguminosarum* the *npr* locus is located downstream of *manX*, a gene coding for an $EIIA^{Man}$ homologue. Thus, *npr* seems to be the link between PTS^{Ntr} and the carbohydrate PTS, modulating the intracellular metabolism. In this work we show a differential regulation driven by these systems. While *ptsN* is required for full activation of ABC transport systems and the high affinity K^+ transporter KdpABC (5), *manX* would interact with the TCA cycle. This work shows that mutants on the PTS^{Ntr} system have a reduced transport rate on different nutrients that need an ATP-dependent transporter. However, although the transport rate of *manX* mutants is not affected, they show a reduced growth on several carbon sources and a compromised oxygen consumption when grown on succinate.

The overall aim of this project is to understand how N-fixing bacteria thrive in different nutritional niches adapting their metabolism. This would allow us to engineer a N-fixing bacteria able to improve nitrogen supply to plants for a sustainable crop yield improvement.

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POSTER 2-3 /LIGHTNING TALK/

The Nod Factor Hydrolase of *Medicago truncatula*: An Example of Symbiosis-related Neofunctionalization

Lan-Yue Zhang[1], Jie Cai[1], Wei Liu[1], Ru-Jie Li[1], Kam-Bo Wong[2], Jiangqi Wen[3], Kirankumar S. Mysore[3], Thomas Boller[4], Zhi-Ping Xie[1], Christian Staehelin[1]

[1] *State Key Laboratory of Biocontrol and Guangdong Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-sen University, East Campus, Guangzhou, China*
[2] *Chinese University of Hong Kong, Shatin, Hong Kong, China*
[3] *The Samuel Roberts Noble Foundation, Ardmore, Oklahoma, USA*
[4] *Botanisches Institut der Universität Basel, Zurich-Basel Plant Science Center, Basel, Switzerland*

The symbiotic association between rhizobia and host legumes depends on lipo-chitooligosaccharidic Nod factors. MtNFH1, the Nod factor hydrolase of *Medicago truncatula*, is a specific enzyme that hydrolytically inactivates Nod factors with a C16:2 acyl chain produced by the microsymbiont *Sinorhizobium meliloti* 1021. MtNFH1 is related to class V chitinases (glycoside hydrolase family 18) but lacks chitinase activity, i.e. does not cleave chitin or chitin oligosaccharides. A homology model of the Nod factor-MtNFH1 interaction suggests a substrate binding pocket with a distinct fatty acid binding cleft formed by loops A and B (1). The amino acid sequence of MtCHIT5b is most similar to MtNFH1. The genes are located in tandem on chromosome 4 of *M. truncatula*. MtCHIT5b is a chitinase that efficiently hydrolyzes chitin oligosaccharides but does not cleave Nod factors. Transcript levels of *MtCHIT5b* are elevated in response to inoculation with the fungal pathogen *Fusarium oxysporum*. Hence, MtCHIT5b shows characteristics of classic chitinases involved in plant defense. Substitution of amino acid residues in either loop A or B of MtCHIT5b results in MtCHIT5b variants that show Nod factor cleaving activity. Remarkably, a single serine-to-proline substitution was sufficient to convert MtCHIT5b into a Nod factor cleaving enzyme. Inversely, MtNFH1 with a corresponding reverse substitution lost the capacity to hydrolyze Nod factors (2). These results are in agreement with a substrate-enzyme model that predicts Nod factor cleavage when the C16:2 acyl chain is placed into a distinct fatty acid-binding cleft. To corroborate the symbiotic role of MtNFH1, we currently characterize mutants with *Tnt1* retrotransposon insertions. We identified mutants with reduced Nod factor cleaving activity and a mutant completely defective in *MtNFH1* expression. Work is ongoing to characterize their symbiotic phenotypes. In summary, our findings support the view that *MtNFH1* evolved from an ancestral *MtCHIT5b* variant by gene duplication and subsequent neofunctionalization. In other words, plant defense related elements were exploited for symbiotic interactions.

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POSTER 2-4 /LIGHTNING TALK/

From Symbiosis to Biotechnology: The Metal Ion-inducible Autocleavage (MIIA) Domain

Jana Schirrmeister[1], Susan Ibe[1], Elisabeth Hoyer[1], Martina Liebmann[1], Susanne Zehner[1], Karim Fahmy[2], Michael Göttfert[1]

[1] *Institute of Genetics, Technische Universität Dresden, Dresden, Germany*
[2] *Institute of Resource Ecology, Helmholtz-Zentrum Dresden-Rossendorf, Dresden, Germany*

Bradyrhizobium japonicum is a Gram-negative soil bacterium and the symbiont of several legumes, e.g. soybean. The plant signal genistein leads to the activation of more than 100 genes. One induced gene cluster encodes a type III secretion system. This is used to transfer effector proteins into the plant cell. The nodulation outer protein NopE1 was proven to be one of these effector proteins (1,2). Bioinformatics analyses revealed that it contains two domains of unknown function (DUF1521), each encompassing about 170 amino acids. NopE1 was expressed in *Escherichia coli* and purified. The protein is cleaved in the presence of calcium. The cleavage site is within the DUF1521 domain, for which we now use the term "metal ion-inducible autocleavage" (MIIA) domain (3,4).

Blast searches unveiled that the MIIA domain is conserved not only in proteins from *B. japonicum* strains but also in proteins of various α-, β-, γ- and δ-Proteobacteria, e.g. the plant growth-promoting endophyte *Burkholderia phytofirmans* PsJN or the coral pathogen *Vibrio coralliilyticus* ATCC-BAA450. The putative protein Vic_001052 from *V. coralliilyticus* contains one MIIA domain. This MIIA domain shows self-cleavage not only in the presence of calcium ions but also in the presence of manganese but not with magnesium ions (4).

Based on the properties of the MIIA domain, we used it as a self-cleaving protein linker. In biotechnology, proteins are often expressed and purified as fusion proteins, which are later cleaved by a costly protease. In contrast, the MIIA domain is an easy low cost tool to release the protein from the fusion partner. Initial tests indicate that cleavage within the MIIA domain is not influenced by the fusion partner. Cleavage is accomplished within minutes on ice and at moderate temperatures. Cleavage is also tolerant towards a pH range from about 5 to 9 (5).

For a more detailed structural characterisation of the MIIA domain, circular dichroism (CD) spectroscopy was used. The MIIA domain is largely unstructured with random coils covering about 75 % of the protein. Upon addition of calcium ions, the percentage of α-helices and β-sheets is increasing. Fluorescence spectroscopy also indicates a strong influence of calcium ions on protein structure.

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POSTER 2-5 /LIGHTNING TALK/

Heterologous Expression of Enzymes of the Nitrogenase Pathway

Laure Decamps, Ivana Djurdjevic, Oliver Einsle

Institute of Biochemistry, Albert-Ludwigs-Universität, Freiburg-im-Breisgau, Germany

Nitrogenase is the only enzyme able to catalyze the reduction of dinitrogen (N_2) to ammonium (NH_3^+) and is therefore essential to fix the nitrogen present in the atmosphere. However, it is not present in eukaryotic organisms, but only in some bacteria and archaea. Hence, plants must rely on interactions with these prokaryotes to obtain nitrogen. Nitrogen availability being a limiting factor for plant growth, making plants independent from these interactions would be a major biotechnological breakthrough.

The transfer of the nitrogen fixation machinery from free-living diazotrophs to model bacteria represents the first step of such a development. We aim to express genes of the nitrogenase pathway from model diazotrophs, for which the lack of genetic tools stands in the way of enzyme bioengineering, to well-characterized laboratory strains. A "minimal set" of 6 genes encoding structural and biosynthetic components (*nifB, nifE, nifN, nifH, nifD, nifK*) has been proposed (1), however so far no nitrogen fixation could be achieved in a heterologous organism with less than 9 genes (the "minimal set" plus *nifX*, *nifV*, and *hesA*) of *Paenibacillus* sp. WLY78 (2). Furthermore, the additional expression of some other accessory proteins may improve the expression and the activity of these enzymes. We intend to first identify the enzymes from various diazotrophs that can be expressed and active in a heterologous organism, then combine them to optimize the nitrogen fixation efficiency. In parallel, we are characterizing these enzymes and setting up reaction assays *in vitro*. This will allow us to engineer these enzymes to increase their activity *in vitro* and *in vivo*.

We have successfully expressed the different components of nitrogenase and of the nitrogenase maturation pathway from a soil free-living organism in a laboratory strain of *Escherichia coli* and combined the expression of some of these components by building new vectors. In parallel to activity assays *in vitro*, we undertook interaction studies to assess the role of accessory proteins that remain undescribed yet.

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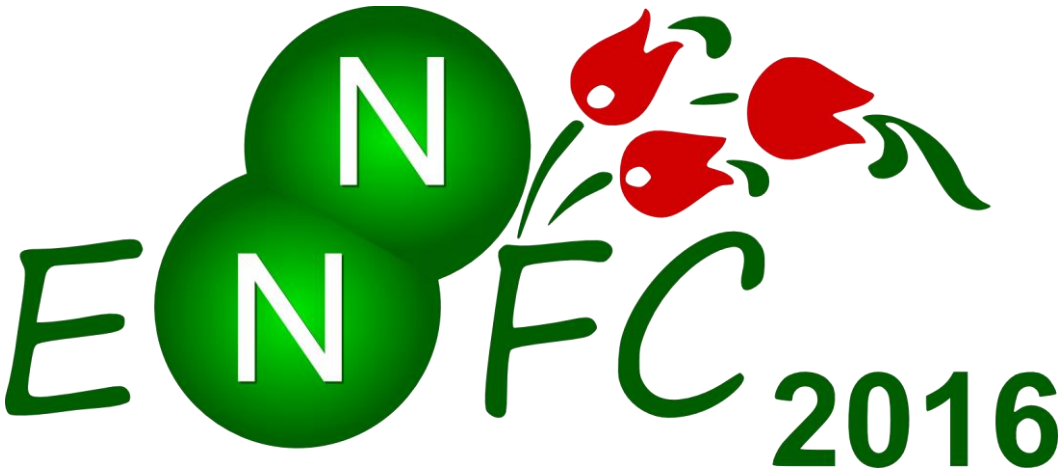
POSTER 2-6 /LIGHTNING TALK/

Exploring the Function of the Inorganic Phosphate Transporter (PiT)-associated Protein in *Sinorhizobium meliloti*

Daniel K. C. Hsieh[1], Vahid Hosseininaveh[2], Rahat Zaheer, Richard Morton[1] and Turlough M. Finan[1]

[1] *Department of Biology, McMaster University, Hamilton, Ontario, Canada*
[2] *Department of Plant Protection, University of Tehran, Karaj, Iran*

Phosphate (Pi) is one of the most important macronutrients required for cellular functions such as signal transduction, membrane lipid dynamics, and nucleotide synthesis. Phosphate is often a limiting nutrient in the environment and its availability can determine the rate of growth of plants and microbes. In *Sinorhizobium meliloti*, there are three Pi transport systems, one of which is a Pit-like transporter, homologous to the well characterized *E. coli* PitA phosphate transporter. However, unlike in *E. coli*, *S. meliloti pit* co-occurs in an operon with *pap* (*orfA*), which encodes a hypothetical protein of unknown function. Previous work has shown that the *pap-pit* operon is negatively regulated by the response regulator PhoB and is therefore expressed under conditions of excess inorganic phosphate. Here, the function of *pap* or *pit-accessory protein (pap*) is investigated through a bioinformatics and mutagenesis approach. Pap orthologs analyzed from bacterial genomes reveal several highly conserved residues within putative PhoU-like motifs. When *S. meliloti* Pap-Pit is the sole phosphate transporter in *S. meliloti* and *E. coli*, substitutions of several conserved residues result in a growth defect in minimal media with inorganic phosphate as the only source of phosphorus. Comparative analyses of the putative Pap-like structures revealed a six alpha-helical bundle similar to that of PhoU, suggesting that Pap may function as a positive regulator of phosphate uptake through the Pit system.



PARALLEL SESSION 3

Infection and Invasion

Chairs: Thomas Ott, Krzysztof Szczyglowski

Diversity of Nodulation and Infection in *Lotus* x *Rhizobium* Combinations Revealed Dynamic Evolutionary Processes

J. Liang and Macarena Marín

Institute of Genetics, Faculty of Biology, University of Munich (LMU), Martinsried, Germany

We have identified a *Rhizobium leguminosarum* isolate that exploits an ancestral intercellular infection pathway in *Lotus*. Intercellular infection represents a more ancient infection mode that precedes the evolutionary innovation that is infection thread formation. Legumes, such as *Arachis, Stylosanthes* and *Aeschynomene* become infected by symbiotic rhizobia through intercellular infection, in which the bacteria enter the root between adjacent epidermal cells (Svistoonoff et al., 2014). This mechanism, in contrast to root hair infection, is root hair- and infection thread-independent and bacteria are directly taken up from the apoplast into root cortical cells. It also differs from crack entry, in which rhizobia exploit gaps in the epidermis of *e.g* Sesbania and form infection pockets that give rise to cortical infection threads (Held et al., 2010). Although *Lotus* becomes infected through root hair infection, there is evidence that it maintains a genetic program for cortical bacterial uptake into single cells from the apoplast (Madsen et al., 2010).

Here we present a *Rhizobium leguminosarum* isolate that exploits this ancestral pathway by colonizing the intercellular spaces and entering cortical cells directly from the apoplast. This *Rhizobium* isolate infected *Lotus* species and ecotypes via an intercellular mechanism; it triggered ecotype-specific nodulation phenotypes (i.e. bumps, nodules and/or multi-lobed nodules) in more than 90 *Lotus japonicus* ecotypes and activated symbiotic signalling in non-nodulating ecotypes. It colonized the root surface and induced typical early responses, such as root hair swelling, branching and curling, but no epidermal infection thread formation. Nodule primordia and later nodules developed. However no trans-cellular infection threads formed in these nodules. Furthermore, intercellular accumulations of bacteria in the apoplast were observed in nodule sections. In these, intact and fully infected nodule cells were present. However, mature nodules rarely became fully colonized and remained white up to 6 weeks post-inoculation. The intercellular infection of *Lotus* seems to be a specific feature of suboptimal matching genotype combinations, as other *Rhizobium* strains induced similar responses, indicating evolutionary novelties.

This constitutes an exceptional system to dissect the uptake of bacteria and the processes associated to their intracellular accommodation inside plant cells. We will use it to identify rhizobial and plant genes required for intercellular infection and intracellular uptake of rhizobia.

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Autoregulation of Infection in the *Sinorhizobium meliloti*-Medicago Symbiosis

Anne M. Garnerone, Fernando G. Sorroche, Mathilda Walch, Catherine Masson-Boivin, Jacques Batut

Laboratoire des Interactions Plantes-Microorganismes (LIPM), Université de Toulouse, INRA, CNRS, Castanet-Tolosan, France

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Autoregulatory mechanisms are essential to the achievement and maintenance of mutualism in beneficial interactions, such as the widespread nitrogen-fixing rhizobium-legume symbiosis. One of the best documented instances is AON (Autoregulation Of Nodulation) that adjusts root nodule number to endogenous and environmental cues in legumes. In most legumes, nodulation is preceded by the infection of root hairs by compatible rhizobia *via* specialized structures called Infection threads (ITs) that initiate in the epidermis before invading root cortex. Despite early evidence that the number of ITs is under control (1), the existence of a specific mechanism controlling infection has remained elusive because of the intricacy between the nodulation and infection processes (2). Here we provide experimental evidence for a systemic autoregulation of infection (AOI) pathway in the *Sinorhizobium meliloti-Medicago truncatula* symbiosis. Contrary to AON, AOI is genetically controlled both by the plant and the bacterium. We provide direct evidence that endosymbiotic bacteria, upon activation of a plant-elicited cAMP-mediated signal transduction cascade (3), down regulate systemically root susceptibility to infection at the pre-infection stage. AOI is thus a new component of the complex regulatory pathway controlling the interaction between rhizobia and legumes.

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Role of SYMbiosis Receptor Kinase (SYMRK) in Synchronising Epidermal Cortical Responses in Root Nodule Symbiosis

Sudip Saha[1], Anindita Paul[1], Laura Herring[2,a], Ayan Dutta[1], Avisek Bhattacharya[1], Sandip Samaddar[1,b], Michael B. Goshe[2] and Maitrayee DasGupta[1]

[1] *Department of Biochemistry, University of Calcutta, Kolkata, India*
[2] *Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, NC, United States*

Present address:
[a] *Proteomics Core, University of North Carolina, Chapel Hill, NC, United States*
[b] *New Chemistry Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India*

The establishment of rhizobia-legume nitrogen fixing root nodule symbiosis (RNS) involves tightly linked developmental events occurring in the root epidermis (notably rhizobial invasion) and the root cortical cells (notably nodule organogenesis and endocytic accommodation of rhizobia). SYMRK is indispensable for activation of RNS at both epidermal and cortical levels and is functionally conserved in legumes. 'Gatekeeper' tyrosine located in the hinge region of SYMRK is phosphorylated both *in vitro* as well as *in planta*. Since gatekeeper phosphorylation was not necessary for activity, the significance of this phosphorylation remained elusive. We demonstrate that substitution of 'gatekeeper' tyr with non-phosphorylatable residues like Phe or Ala significantly affected autophosphorylation on activation segment, P+1 and β3-αC loops of SYMRK. Overexpression of both Wildtype and 'gatekeeper' substituted SYMRK kinase domains (SYMRK-kd) hyperactivated spontaneous nodule organogenesis in TR25 (symrk null) indicating that both ectodomains and phosphorylations on the above targets were disposable for inducing cortical cell division and nodule organogenesis. Unlike the wildtype SYMRK-kd, the 'gatekeeper' substituted SYMRK-kds failed to restore any rhizobial infection in TR25 suggesting the phosphorylations on activation segment, P+1 and β3-αC loops of SYMRK was important for infection events. Alongside, the same 'gatekeeper' mutations in full length WT also failed to restore proper symbiotic features in TR25 where rhizobial invasion in epidermis and nodule organogenesis in cortex was unaffected but rhizobia remain restricted in the epidermis in infection threads migrating parallel to the longitudinal axis of the root resulting in extensive infection patches at the nodule apex. Thus 'gatekeeper' phosphorylation on SYMRK is critical for synchronizing epidermal infection events with the /cortical developental processes in RNS.

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The Unique *Brush* Allele Reveals Redundancy in a Cluster of Channel Proteins during Root Development and Infection by Rhizobia

David M. Chiasson, K. Haage, K. Sollweck and M. Parniske

Faculty of Biology, Genetics, University of Munich (LMU), Martinsried, Germany

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The *brush* mutant was previously identified in a screen for *Lotus japonicus* plants impaired in nodulation (1). *brush* plants have short, thick roots and are compromised in infection by rhizobia. The causative recessive mutation has been mapped to *LjCNGC20*, encoding an uncharacterized cyclic nucleotide-gated channel protein. Phenotypic and genetic analysis have revealed that *brush* is impaired in infection thread development and lies downstream of the common symbiosis signaling cascade. Complementation experiments have demonstrated that overexpression of *LjCNGC20* is required to rescue *brush*. Further, knockdown of the *brush* allele in the mutant rescues the infection and root development phenotypes and overexpression of *brush* blocks root development entirely. Collectively these results point to a dosage-dependent negatively interfering function for *brush*. A null *LjCNGC20* allele exhibited no apparent root or infection phenotypes, suggesting possible redundancy with other *CNGC*s. Interestingly, *LjCNGC20* resides in a genomic cluster of *CNGC*s that has expanded specifically in the legume lineage. In line with potential redundancy amongst CNGCs, overexpression of either of the three other cluster members rescues the *brush* mutant. *LjCNGC20* contains a point mutation in *brush*, leading to an amino acid exchange in the N-terminus of the protein. This mutation altered protein-protein interactions in yeast two-hybrid assays leading us to conclude that *brush* has acquired a novel function. The unique recessive antimorphic *brush* allele has thus enabled insight into the function of a redundant family of channels during root development and infection by rhizobia.

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POSTER 3-1 /LIGHTNING TALK/

NF-Y TFs as Key Regulators of Nodule Development and Infection

Arnaud Bethencourt, Agnès Lepage, Farid Regad, Carolina Rípodas, Maël Baudin*, Lisa Frances, Pascal Gamas, Fernanda de Carvalho-Niebel and Andreas Niebel

Laboratoire des Interactions Plantes-Microorganismes (LIPM), CNRS, UMR2594, Castanet-Tolosan, France *andreas.niebel@toulouse.inra.fr*

* *Present address: Department of Plant and Microbial Biology, University of California, Berkeley, California, United States*

Plants belonging to the legume family are able to interact symbiotically with nitrogen fixing bacteria named rhizobia. This symbiotic interaction leads to the formation of a new organ on the roots of the host plants, called the root nodule, inside which atmospheric nitrogen is fixed for the benefit of the plant. Evidence for a key role for NF-Y transcription factors (TFs) during symbiotic infection and legume nodule development has gradually emerged (Combier et al., 2006; Zanetti et al., 2010; Soyano et al., 2013; Laloum et al., 2014; Laporte et al., 2014; Xiao et al., 2014; Baudin et al., 2015). NF-Y are CCAAT-box binding TFs also called nuclear factor Y (NF-Y) (Laloum et al., 2013). The heterotrimeric NF-Y is composed of the DNA-binding subunit NF-YA associated with two histone-like subunits NF-YB and NF-YC. We are trying to understand the mechanisms by which NF-Y TFs control nodule development and infection. We have shown that *MtNFYA1*, *MtNF-YB16, and MtNF-YC2*, form the main NF-Y trimer, active during nodulation in *Medicago truncatula.*

We will present data on the identification and characterization of their target genes using ChIP-seq and RNA-seq and also their interacting proteins partners.

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POSTER 3-2 /LIGHTNING TALK/

SCARN a Novel Class of SCAR Protein that is Required for Root-hair Infection During Legume Nodulation

Liping Qiu[1], Jie-shun Lin[1], Ji Xu[1], Shusei Sato[2,3], Martin Parniske[4], Trevor L. Wang[5], J. Allan Downie[5], Fang Xie[1]

[1] *National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai, Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China*
[2] *Kazusa DNA Research Institute, Kisarazu, Japan*
[3] *Graduate School of Life Sciences, Tohoku University, Sendai, Japan*
[4] *University of Munich LMU, Faculty of Biology, Martinsried, Germany*
[5] *John Innes Centre, Norwich, United Kingdom*

Rhizobial infection of legume root hairs requires a rearrangement of the actin cytoskeleton to enable the establishment of plant-made infection structures called infection threads. In the SCAR/WAVE (Suppressor of cAMP receptor defect/WASP family verpolin homologous protein) actin regulatory complex, the conserved N-terminal domains of SCAR proteins interact with other components of the SCAR/WAVE complex. The conserved C-terminal domains of SCAR proteins bind to and activate the actin-related protein 2/3 (ARP2/3) complex, which can bind to actin filaments catalyzing new actin filament formation by nucleating actin branching. We have identified, SCARN (SCAR-Nodulation), a gene required for root hair infection of *Lotus japonicus* by *Mesorhizobium loti*. Although the SCARN protein is related to Arabidopsis thaliana SCAR2 and SCAR4, it belongs to a distinct legume-sub clade. We identified other SCARN-like proteins in legumes and phylogeny analyses suggested that SCARN may have arisen from gene duplication and acquired specialized functions in root nodule symbiosis. Mutation of SCARN reduced formation of infection threads and their extension into the root cortex and slightly reduced root-hair length. However we observed no effect of *scarn* mutations on trichome development or on the early actin cytoskeletal accumulation that is normally seen in root hair tips shortly after *M. loti* inoculation, distinguishing them from other symbiosis mutations affecting actin nucleation. The C-terminal domain of SCARN binds to ARPC3 and ectopic expression of the N-terminal SCAR-homology domain (but not the full length protein) inhibited nodulation. In addition, we found that SCARN expression is enhanced by *M. loti* in epidermal cells and that this is directly regulated by the NODULE INCEPTION (NIN) transcription factor.

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POSTER 3-3 /LIGHTNING TALK/

Host Cell Reprogramming for Rhizobial Root Infection

A. Kelner[1], L. Frances[1], M. R. Cerri[2], M. Beck[1], M. F. Guerrero-Molina[1], M. Chabaud[1], D. G. Barker[1], G. E. Oldroyd[3], J. Fournier[1] and F. de Carvalho-Niebel[1]

[1] *Laboratory of Plant Microbe Interactions (LIPM), INRA, CNRS, Université de Toulouse, Castanet-Tolosan, France*
[2] *Current address: Faculty of Biology, University of Munich (LMU), Institute of Genetics, Grosshaderner Str. 2-4, D-82152 Martinsried, Germany*
[3] *John Innes Centre, Norwich NR4 7UH, United Kingdom*

The development of a functional nitrogen-fixing nodule in legumes depends on a successful molecular dialogue between the plant and respective rhizobial partner before the bacteria can enter the host root. Rhizobial infection then occurs *via* a host defined pathway within newly formed apoplastic tubular structures (infection threads), which involves coordinated reprogramming of adjacent root cell layers. We recently demonstrated that two closely-related ERF-type transcription factors (ERN1 and ERN2) are essential for early rhizobial infection of *Medicago truncatula* roots (1,2,4). While *ERN1* and *ERN2* act in concert to regulate early steps of root hair epidermal cell entry, only ERN1 is essential for root cortical cell infection. In addition to genetic approaches, *in vivo* cell imaging has revealed that ERN1 sequentially accumulates in the nuclei of root cells undergoing infection (3), which underlines the importance of cell-specific reprogramming during these early steps of bacterial entry. We now focus on the understanding of the nuclear reprogramming that takes place at this particular early stage of infection by using a variety of complementary strategies. This includes the analyses of gene expression changes in single and double mutant lines to identify downstream genes directly dependent on both ERN1/ERN2 factors and also new *in vivo* cell imaging approaches combined with symbiotic mutants that allow us to follow precise nuclear changes that occur during this early symbiotic stage.

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POSTER 3-4 /LIGHTNING TALK/

The ERF Required for Nodulation1 (ERN1) Transcription Factor is Required for Root Nodule Infection in *Lotus japonicus*

Yasuyuki Kawaharada[1,2], Simon Kelly[1,2], Niels Sandal[1,2], Euan K. James[3], Simona Radutoiu[1,2] and Jens Stougaard[1,2]

[1] *Centre for Carbohydrate Recognition and Signalling, Aarhus University, Denmark*
[2] *Department of Molecular Biology and Genetics, Aarhus University, Denmark*
[3] *The James Hutton Institute, Invergowrie, United Kingdom*

The symbiotic association between legume and rhizobia is accomplished through two developmental processes: The rhizobial infection and nodule organogenesis. The final outcome of the interaction is the formation of root nodules within which the rhizobia reside. Several legume transcription factors have been identified and shown to be important for this symbiosis. For example, ERF Required for Nodulation (ERN)1, ERN2 and ERN3 are transcription factors containing an APETALA2/Ethylene Responsive Factor (AP2/ERF) domain, that were identified in *Medicago truncatula*. In this study, we characterise the role of ERNs in the determinate nodule forming model legume *Lotus japonicus,* and identify factors involved up- and down-stream of these transcription factors.

Ern1 and *Ern3* homologs have been identified in the current *L. japonicus* genomic sequence data (v3.0), but no *Ern2* homolog could be identified. Four *ern1* allelic mutants containing LORE1 retrotransposon insertions, show are impaired in infection thread formation when inoculated with the symbiotic bacteria *Mesorhizobium loti*. However, it was observed that they form effective nodules via a crack-entry colonisation mechanism 3 to 4 weeks post-inoculation. The nodules formed, *ern1* mutants exhibited abnormal and peg-type cortical infection threads. These observations suggest that ERN1 is important for forming infection threads in both root hairs and nodules. Spontaneous nodulation assays using autoactive CCaMK (T265D) transgenic roots and exogenous cytokinin treatment, revealed that ERN1 additionally regulates nodule organogenesis. RT-qPCR and *Ern1* promoter GUS analyses revealed that *Ern1* expression is induced by Nod factor, and this expression is localised to the susceptible zone of the epidermis at early stages of symbiosis and in the cortex at later stages during development of nodule primordia. In addition, we have identified that the *Epr3* encoded Expolysaccharide receptor[1], and several other genes are induced directly or indirectly by ERN1[2,3].

Results that position the ERN1 transcription factor in the *Lotus* symbiotic signalling genetic pathway will be presented and discussed.

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POSTER 3-5 /LIGHTNING TALK/

Regulation of *Lotus japonicus ERN1* by the CCaMK/CYCLOPS Complex Constitutes a Central Step in the Transcription Factor Network Controlling Bacterial Accommodation

Marion R. Cerri[1], Quanhui Wang[2], Xiaolin Li[2], Paul Stolz[1], Jessica Folgmann[1], Lisa Frances[3], Katja Katzer[1], Fernanda de Carvalho-Niebel[3], Trevor L. Wang[4], Allan Downie[4], Fang Xie[2] and Martin Parniske[1]

[1] *Faculty of Biology, University of Munich (LMU), Institute of Genetics, Martinsried, Germany*
[2] *National Key Laboratory of Plant Molecular Genetics, CAS Center for Excellence in Molecular Plant Sciences, Institute of Plant physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China*
[3] *Laboratory of Plant-Microbe Interactions (LIPM), Centre National de la Recherche Scientifique (CNRS, UMR2594), Institut National de la Recherche Scientifique (INRA), Castanet-Tolosan, France*
[4] *John Innes Centre, Norwich, United Kingdom*

The nitrogen-fixing root nodule symbiosis of legumes contributes significantly to protein nutrition of humans and animals. The mechanism of bacterial entry into plant cells and the regulation of this process is a key feature of this interaction. In order to study the genetic basis of the infection process, a forward genetic screen was performed in search for infection mutants of the model legume *Lotus japonicus* [(1)]. We identified an infection-related gene from a *L. japonicus* mutant through genetic analysis of an EMS-induced mutant line impaired in infection thread formation. Based on phylogenetic analysis, syntheny and complementation assay, we confirmed that the identified gene is an orthologue of *ERF Required for Nodulation 1* (*ERN1),* encoding an AP2/ERF-type DNA-binding domain carrying transcriptional factor, previously identified in *Medicago truncatula* [(2,3)]. In contrast to *M. truncatula*, *L. japonicus* carries only one gene copy within the *ERN* genomic cluster. A detailed phenotypic characterization of two independent *ern1* mutant lines in *L. japonicus* revealed that *LjERN1* fully controls bacterial entry via infection thread initiation and progression. Interestingly, the phenotypic defect of *Ljern1* displays common features but also differences compared to that of the previously characterized *M. truncatula ern1* or *ern2* mutants [(2,4)]. Importantly, we discovered that the CCaMK/CYCLOPS complex regulates positively *ERN1* expression. The predicted CYCLOPS binding site on the *ERN1* promoter was confirmed by transactivation assays and DNA-protein interaction assays *in vitro*. Our data reveal a central node of the transcriptional network that controls bacterial entry into plant cells.

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POSTER 3-6 /LIGHTNING TALK/

Policing the Gate: Can Pea Plants Stop Rhizobial Cheats from Entering?

Annet Westhoek[1, 2], F. Rehling[3], P. S. Poole[1], L. A. Turnbull[1]

[1] *Department of Plant Sciences, University of Oxford, Oxford, United Kingdom*
[2] *Systems Biology Doctoral Training Centre, University of Oxford, United Kingdom*
[3] *Department of Ecology, Philipps University Marburg, Marburg, Germany*

Legumes form symbioses with nitrogen-fixing soil bacteria called rhizobia. An intricate signalling process allows rhizobia to infect plant roots and form nodules. Inside nodules, rhizobia fix atmospheric nitrogen into ammonia and provide it to the plant (1). Rhizobial strains vary widely in how much nitrogen they provide and this affects crop yields (2). Despite some evidence to the contrary, there have been recent claims that legumes exert 'partner choice' and selectively form symbioses with rhizobia that provide more nitrogen (3,4). We tested whether peas exert such partner choice.

As many traits influence the ability of rhizobia to form nodules, the only unbiased test of partner choice requires the use of strains that differ in their ability to fix nitrogen, but nothing else. We developed sets of wild-type nitrogen-fixing strains and their respective *nifH* mutant non-fixing strains. Strains were distinguished using chromosomal *gusA* and *celB* marker genes and were otherwise completely isogenic. Peas were inoculated with different ratios of fixing to non-fixing strains. We found that the percentage of nodules containing the fixing strain exactly reflected the percentage of the fixing strain in the inoculum. We therefore found no evidence for partner choice.

Our results demonstrate that pea plants cannot exercise partner choice. This emphasizes the essential role of plant sanctions for plant and rhizobial fitness. In sanctioning, plants allocate fewer resources to established nodules providing little nitrogen (5). Ongoing work will focus on how such sanctions affect crop yields and populations of effective and less effective rhizobia in the soil.

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POSTER 3-7 /LIGHTNING TALK/

Molecular Control of Receptor Mobility Shifts during Rhizobial Infection

T. F. Stratil, P. Liang, C. Popp, S. M. Marín, J. Folgmann, Thomas Ott

Institute of Genetics, Ludwig-Maximilians-Universität München, Planegg-Martinsried, Germany

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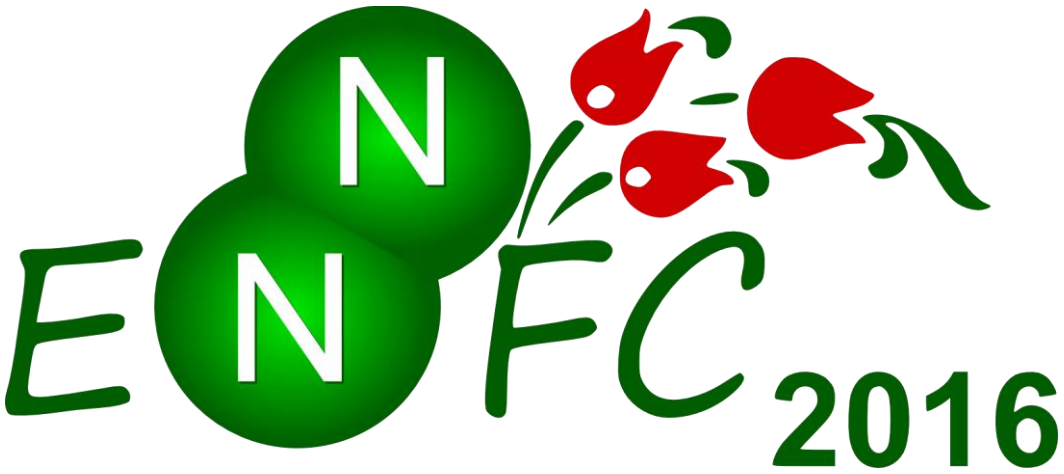
Intracellular colonization of plant cells by symbiotic bacteria is a critical step for the host that requires stringent surveillance circuits at the plasma membrane to keep exclusive control over the infection process. Accumulating evidence suggests that such perception and signal transduction complexes are pre-formed in membrane compartments such as mesoscale membrane domains (MDs). However, neither the existence of pathway-specific MDs nor their controlled assembly has been demonstrated. Here, we unravelled the sequential organization of membrane-resident signalling proteins that are indispensible for the intracellular infection of *Medicago truncatula* roots by symbiotic bacteria. We identified actin, the flotillin FLOT4, the remorin SYMREM1 and the entry receptor LYK3 as essential molecular building blocks that are required and sufficient for the assembly of an infection-related MD in vivo. In addition we unravelled the mechanism that leads to the lateral immobilization of the LYK3 receptor in this MD upon rhizobial infection.



PARALLEL SESSION 4

Interplay of Nitrogen-fixing and Mycorrhizal Symbioses

Chairs: Allan Downie, Caroline Gutjahr

Parasponia and Trema Comparative Genomics to Provide Insight in an Evolutionary Trajectory towards Rhizobium Symbiosis

R. Holmer[*1,2], R. van Velzen[*1], F. Bu[1], L. Rutten[1], L. Santuari[1], T. Sharma[1], T. Wardhani[1], W. Kohlen[1], A. van Zeijl[1], S. Smit[2] and Rene Geurts[1]
*These authors contribute equally.

[1] *Wageningen University, Department of Plant Science, Laboratory of Molecular Biology, Wageningen, The Netherlands*
[2] *Wageningen University, Department of Plant Science, Laboratory of Bioinformatics, Wageningen, The Netherlands*

Nitrogen fixing root nodules evolved up to ten times in the plant kingdom, with either rhizobium or Frankia as diazotrophic microsymbiont. Comparative studies revealed that evolution of this trait -at least in part- was guided by genetic constraints. However, despite extensive research especially in legumes, the genetic changes allowing plants to establish a nitrogen fixing root nodule endosymbiosis has not been identified[1]. We established a comparative system of two closely related *Cannabaceae* species that differ in occurrence of rhizobium root nodules; namely symbiotic *Parasponia andersonii* and its non-symbiotic sister species *Trema orientalis*. Genome sequencing and annotation uncovered ~36-k gene models for each species. Pairs of orthologous genes were identified by using a combination of whole genome alignment and orthology grouping. This allowed us to conduct comparative interspecific transcriptomics in *Parasponia* and *Trema*. We focussed on over hundred genes that in legumes have a symbiotic function, and found gene copy number variation in 9 out of 150 orthology groups, 3 of which are in symbiotic LysM-type receptor kinases. Additionally, we identified a *Parasponia* specific splice variant in a key transcription factor. Comparative interspecific transcriptomics on all symbiosis genes in different root tissues revealed specific subclasses of genes that are differentially expressed between both species. This suggests that these (putative) symbiosis genes evolved regulatory adaptations. In a symbiotic context, expression of many of these putative symbiosis genes was enhanced. This suggests that during evolution of rhizobium symbiosis in legumes and *Parasponia* orthologous genes were recruited to function in root nodules. Our results demonstrate that the Parasponia-Trema comparative system provides novel platform to study evolution of rhizobium nitrogen fixing symbiosis. Adapting both plant species to experimental systems will allow comparative studies to underpin the genetic changes that are essential to establish a rhizobium N_2-fixing symbiosis.

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Arbuscular Mycorrhiza Development in Pea Mutants Impaired in Early Nodulation Genes including Putative Orthologs of *NSP1* and *NSP2*

Oksana Shtark[1,2], Jaroslava Fedorina[1], Anton Sulima[1], Alexander Zhernakov[1], Marina Kliukova[1,2], Maria Khairullina[2], Alexander Pinaev[1], Igor Tikhonovich[1,2] and Vladimir Zhukov

[1] *All-Russia Research Institute for Agricultural Microbiology, Pushkin, Saint-Petersburg, Russian Federation*
[2] *Faculty of Biology, Saint-Petersburg State University, Saint-Petersburg, Russian Federation*

Pea (*Pisum sativum* L.) forms both arbuscular mycorrhiza (AM) and nitrogen-fixing root nodules (RN). These two endosymbioses improve plant nutrition, enhance plant resistance to biotic and abiotic stresses, and increase crop yield and quality (1). Pea regulatory symbiotic (*SYM*) genes, which control AM development, are still insufficiently characterized. In this study, AM phenotype of a series of non-nodulating pea mutants carrying mutations in four *SYM* genes was analyzed at the early stages of colonization by *Rhizophagus irregularis*. Our data, which display both abundance of external mycelium attached to the root surface and parameters of internal fungal colonization, indicate that all pea genes analyzed are essential for AM development. Mutations in *SYM7* (putative ortholog of *NSP2*, 'nodulation signaling pathway 2'(2, 3)), *SYM11*, and *SYM14* genes resulted in a considerable increase in root surface colonization and a substantial decrease in internal colonization as compared to corresponding wild-type pea lines (*wt*). We also provide evidence that *SYM7* is essential for arbuscule development. In contrast, plants mutated for *SYM34* gene displayed strongly reduced root surface colonization; also they had strongly reduced internal colonization after 10 days of growth, but did not differ from *wt* 10 days later. The described AM phenotype suggested that the pea *SYM34* gene is an ortholog of *NSP1* ('nodulation signaling pathway 1' (4)), and early stop codons were in fact detected in a *Medicago truncatula NSP1* homologous sequence of two *sym34* mutants. In addition, our hypothesis was supported by a co-segregation analysis. Both *NSP1* and *NSP2* are GRAS-family transcription factors indispensable for RN development (2, 4); also they are involved in AM formation and activation of biosynthesis of AM-fungal growth regulators such as strigolactones (*NSP1* and *NSP2*) and cutin monomers (*NSP2*) (5). With this in mind, a possible explanation for the different AM phenotypes of the *sym34* (=*Psnsp1*) and *sym7* (=*Psnsp2*) mutants will be given.

This work was supported by the Grants of RSF (14-24-00135 и 16-16-00118), RFBR (14-04-01442, 15-29-02737, and 16-04-01859) and SPBU Grant (1.37.534.2016).

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A CCaMK-CYCLOPS-DELLA Complex Regulates Transcription of *RAM1* in Arbuscular Mycorrhiza

Priya Pimprikar, Samy Carbonnel, Michael Paries, Katja Katzer, Verena Klingl, Monica Bohmer, Leonhard Karl, Martin Parniske, Caroline Gutjahr

Faculty of Biology, Genetics, University of Munich (LMU), Biocenter Martinsried, Martinsried, Germany
caroline.gutjahr@ lmu.de

Establishment of both arbuscular mycorrhiza and root nodule symbiosis requires the common SYM gene *CYCLOPS*, which encodes a transcriptional activator [1, 2]. CYCLOPS is activated through phosphorylation by the calcium and calmodulin-dependent kinase CCaMK and can then induce the nodulation-specific gene *NODULE INCEPTION* (*NIN*) by directly binding to a *cis*-element (*CYC-RE*) in the *NIN* promoter [2]. However, direct AM-specific CYCLOPS-targets had not been described.

We found that CYCLOPS is required for activating the AM-specific gene *RAM1*, which is involved in arbuscule development. When co-expressed with gain of function (GOF) CCaMK in *Nicotiana benthamiana* leaves it transactivates the *RAM1* promoter by direct binding to the *cis*-element *AMCYC-RE*, which differs from the *CYC-RE* in the *NIN* promoter [3].

In AM symbiosis, *cyclops* mutants allow formation of intraradical hyphae in the cortex but not of arbuscules [4]. A similar phenotype is observed when DELLA proteins, repressors of gibberellin (GA) signaling, are removed from the system by mutation of the corresponding genes or by treatment with GA [5], suggesting that both CYCLOPS and DELLA are required for arbuscule formation. We found that CYCLOPS physically interacts with DELLA and co-expression of DELLA with GOF-CCaMK and CYCLOPS in *N. benthamiana* leaves increases transactivation of the *RAM1* promoter. Ectopic expression of *RAM1* in *Lotus japonicus* roots supports arbuscule formation in a *cyclops* mutant and in presence of inhibitory GA, confirming that *RAM1* acts downstream of the CYCLOPS-DELLA complex [3]. We reveal a transcription factor complex that integrates symbiosis and GA signaling and possibly participates in adjusting symbiosis development with the plant physiological state.

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Differential Host-Selection Behaviour in the *Rhizobium leguminosarum* bv. Viciae - Legume Symbiosis

Beatriz Jorrín[1], Juan Imperial[1,2]

[1] *Centro de Biotecnología y Genómica de Plantas (UPM-INIA), Madrid, Spain*
[2] *Consejo Superior de Investigaciones Científicas (CSIC), Spain*

The establishment and maintenance of the symbiotic partnership results from a molecular conversation that guarantees specificity and guides the co-development of both organisms during root nodule formation. *Rhizobium leguminosarum* bv. viciae is a member of the α-Proteobacteria that can establish effective symbioses with members of the Fabeae legume tribe (*Pisum, Lathryrus, Lens* and *Vicia*). Previous studies have suggested that, although all *R. leguminosarum* bv. viciae isolates can effectively nodulate all Fabeae, different Fabeae select specific genotypes of rhizobia from those available in soil.

The aim of the present work was to characterize the genomic, genetic and molecular bases of the selection of specific *R. leguminosarum* bv. viciae genotypes by different host legumes (*Pisum sativum*, *Lens culinaris*, *Vicia faba* and *V. sativa*) from a well-characterized agricultural soil.

We established a population genomics methodology based on pooled DNA samples (Pool-Seq) from *R. leguminosarum* isolates obtained from different sources: legume plant hosts used as rhizobial traps (*P. sativum*, *L. culinaris*, *V. sativa* and *V. faba*), as well as the isolation of *R. leguminosarum* directly from soil. This approach allowed us to confirm the hypothesis that different plant hosts select specific subpopulations of rhizobia from the available population present in the soil.

We also set out to characterize the indigenous *R. leguminosarum* soil population avoiding plant selection, in order to compare it with previously characterized, host-selected subpopulations from this soil. As a side result, we uncovered a large number of previously uncharacterized, non-symbiotic rhizobia that contribute to the population pangenome. Host preference for specific genotypes was especially relevant in the case of pea plants. They selected a *R. leguminosarum* population significantly different from that present in the soil. Quite on the contrary, lentil and fava bean plants did not show a significant genotype selection, and their nodule rhizobial populations reflected that present in soil (after one life cycle). Vetch plants revealed a certain genotypic preference, but not as substantial or as important as that from pea plants.

Given that plants can differentially select rhizobial genotypes and that viable rhizobia of those genotypes are released into soil after nodule senescence, we hypothesized that, in natural conditions after numerous cycles of selection-release, most nodules would be occupied by the preferred genotype/s in each plant. We experimentally tested this hypothesis in a mesocosm experiment aimed at mimicking these field conditions. We were able to demonstrate that the different plant hosts employed (*P. sativum*, *L. culinaris*, *V. faba* and *V. sativa*) selected different genotypes from those available in the P1 soil, and that the basis of selection was different for different plants. Pea and fava bean plants strongly selected for specific genotypes, but in different ways. Pea nodules were colonized by strains endowed with a large set of genes probably implicated in rhizospheric fitness, irrespective of the symbiotic genotype they harboured. This suggestion should be confirmed by *in situ* transcriptomic studies. Fava bean plants restricted their selection to a specific symbiotic genotype that was not always localized in the same symbiotic plasmid, or within the same chromosomal background. No hard conclusions could be obtained for vetch, although we suggest that this plant might behave dually, either as a selective host if a given genotype results in a rhizospheric advantage (such as in vetch$_B$ subpopulation), or as a non-selective host, with its nodules reflecting the genotypic diversity present in soil (such as in vetch$_A$ subpopulation). This last case was the situation found for lentils; none of the three subpopulations isolated from lentil nodules (initial lentil, lentil$_A$ and lentil$_B$) differed significantly, either among themselves, regardless of the number of plant selection cycles, or with respect to the initial soil population.

POSTER 4-1 /LIGHTNING TALK/

Do You Want to Join the Complex? Towards the Identification of New CCaMK/CYCLOPS Interactors

A. Sandré, C. Cathebras, P. Bellon, Rosa Elena Andrade Aguirre, X. Gong, M. Parniske

Faculty of Biology, Genetics, University of Munich (LMU), Martinsried, Germany

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During evolution plants recruited genes previously involved in arbuscular mycorrhiza (AM) symbiosis to establish a new interaction with nitrogen-fixing bacteria called root nodule (RN) symbiosis (1). The establishment of these symbioses is triggered by a signaling transduction pathway featuring perinuclear calcium oscillations, conceptually deciphered by CCaMK (1, 2). CCaMK phosphorylates CYCLOPS (2, 3) and forms a protein complex (CCaMK/CYCLOPS), that transcriptionally activates downstream genes required for specific developmental responses in AM and RN symbiosis (3, 4). Additionally, we found that this complex is involved in regulating lateral root formation in *Lotus japonicus*.

Although this complex has a strategic place upstream different pathways, little is known about how those are differentially induced. Moreover, rice orthologs of CCaMK and CYCLOPS are able to restore nodulation of corresponding legume mutants (2, 5), indicating that the acquisition of the ability to induce the transcriptional network required for root nodule symbiosis does not rely on functional adaptation of CCaMK or CYCLOPS.

Recent results indicate that the CCaMK/CYCLOPS interaction appears to be the core of a larger dynamic complex. For example, DELLA is recruited into the CCaMK/CYCLOPS complex to activate transcription of *RAM1* during AM (4). It is likely that additional components of the complex may help to determine the correct transcriptional responses appropriate for different symbiotic and developmental processes. The CCaMK/CYCLOPS interactome and the mechanisms by which novel interactors contribute to specific transcriptional responses required for the two distinct symbioses and lateral root development are under investigation.

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POSTER 4-2 /LIGHTNING TALK/

The Process of Bacteroid Differentiation in Pea (*Pisum sativum* L.) is Controlled by Symbiotic Genes that Regulate the Expression of the NCR Gene Family

Igor A. Tikhonovich[1,2], Marina S. Kliukova[1,2], Olga A. Kulaeva[1], Alexander I. Zhernakov[1], Vladimir A. Zhukov[1]

[1] *All-Russia Research Institute for Agricultural Microbiology, St.-Petersburg, Russia*
[2] *Saint-Petersburg State University, St.-Petersburg, Russia*

The ability of leguminous plants to control the symbiotic bacteria in nodules is based on their capability to trigger the differentiation the bacteria into the symbiotic form - bacteroids. In Legumes of IRLC clade, this differentiation is promoted and mediated by short plant peptides named NCR (nodule-specific cysteine-rich) peptides (1, 2). NCR peptides are highly divergent in amino acid composition, so each species possesses its own spectrum of NCR peptides. The gene family composed of more than 600 members encoding NCR peptides has recently been characterized in *Medicago truncatula* (2, 3). In pea, only some members of this gene family are known (characterized as early nodulins) (4).

The development of the "next generation sequencing" (NGS) methods along with the availability of the unique pea mutants made it possible to describe the gene family encoding NCR peptides in pea and to study the genetic control over the NCR gene family expression. Using both the publically available and original data on pea nodule transcriptome sequencing (5), we found more than 200 nodule-specific transcripts encoding peptides that belong to NCR family. The transcripts were classified into groups according to expression level in 12-, 21- and 28-days old wild type nodules (based on original RNAseq data). Then, also using RNAseq, their expression was evaluated in nodules of several symbiotic mutant lines with defects in nodule development and functioning. Virtually all NCR genes were downregulated in *sym31* and *sym33* mutants (forming ineffective nodules with no signs of bacteroid differentiation). Only "late" NCR genes were downregulated in *sym40* and *sym42* mutants (forming nodules with abnormal morphological differentiation of bacteroids), whereas the "early" NCR genes were either upregulated or unchanged. Almost only "early" NCR genes were significantly upregulated in *sym26* and *sym27* mutants (forming nodules with prematurely degraded symbiotic structures). The expression level of ten selected NCR genes was evaluated by real time PCR on early stages of nodule development, confirming different regulation of expression for different groups of NCR genes in pea.

The fact that mutant phenotype is associated with mis-expression of only some NCR genes points at possible key role of the particular NCR peptides in regulation of bacteroid differentiation in pea. In general, the different expression of NCR genes in mutants with different degree of bacteroid differentiation allows us to outline the scheme of the plant control over the microsymbiont's fate during nodule development.

This work was supported by the Russian Science Foundation [grant number 14-24-00135].

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POSTER 4-3 /LIGHTNING TALK/

Expression of a Rhizobial Efflux System and its Associated Transcriptional Regulator during Nodule Development

A. Welker, S. Krysenko, D. Mitić, M. Göttfert and Anna Zdyb

Institute of Genetics, Technische Universität Dresden, Dresden
Anna_Maria.Zdyb@tu-dresden.de

Symbiotic nitrogen-fixing bacteria are exposed to various toxic plant compounds during the establishment of a successful symbiosis. Bacteria are equipped with several efflux systems that allow the extrusion of harmful chemicals encountered in their environment. Rhizobial genome sequences reveal the presence of several efflux systems belonging to different families. In the alfalfa symbiont *Sinorhizobium meliloti* strain 1021, 14 efflux systems have been identified (1). In transcriptome analyses, the genes *SMc03167* and *SMc03168* – the deduced proteins are similar to the multi-drug resistance proteins EmrB and EmrA of *E. coli*, respectively – were reported to be inducible by luteolin, a plant signal known to induce nodulation genes (2). Using a transcriptional *emrA-gusA* fusion, we demonstrated that the gene is inducible by several flavonoids also by quercetin, which is not an inducer of nodulation genes. This suggests that the gene is not regulated directly by NodD, which is the activator of nodulation genes. Upstream of *emrA*, a TetR-type regulator (EmrR) is encoded. EmrR binds to palindrome-like sequences within the *emrA-emrR* intergenic region (3). Our investigation revealed that *emrR* is also inducible by apigenin. After integration of the *emrR-lacZ* fusion into an *emrR* mutant background, the fusion was no longer inducible by apigenin. However, the expression level in the non-induced strain was significantly higher than in the wild-type background. This suggests that EmrR acts as a repressor, which regulates the transcription of *emrAB* and of its own gene. Interestingly, a mutation of *emrR* but not of *emrA*, impaired symbiosis with alfalfa (3, 4 and unpublished results). This might indicate that a proper regulation of *emrAB* is essential for the interaction of *S. meliloti* with alfalfa. To address this issue, we used reporter gene fusions of *emrA* and *emrR* and studied their expression in nodules of alfalfa and *Medicago truncatula*. Preliminary results indicate that EmrA is expressed in the infection zone of alfalfa and *M. truncatula* nodules. Expression of EmrR was detected in the infection zone and also throughout the fixation zone. There was no expression detected in the infection threads of mature nodules neither during the initial infection thread formation. This result suggests that the expression of this efflux system is only triggered at a specific point of nodule development.

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POSTER 4-4 /LIGHTNING TALK/

Rhizobial Competition: Getting to the Root of the Problem

Marcela A. Mendoza-Suárez, B. A. Geddes, C. Sánchez-Cañizares, P. S. Poole

Department of Plant Sciences, University of Oxford, Oxford, United Kingdom

The symbiosis formed between plants of the legume family with nitrogen-fixing bacteria called rhizobia is of major global importance in agricultural systems [1]. As free-living cells in the soil, competition amongst them is crucial, as the most competitive rhizobial strain will be the one able to infect the legume host and form a nodule. Unfortunately, competitiveness and effectiveness, which is the ability of rhizobia to reduce N_2 to ammonia and transfer it to the plant, are independent rhizobial traits. Therefore, legumes tend to become nodulated by highly competitive but not necessarily effective rhizobial strains, resulting in a less optimal plant growth [2]. This is a major problem for nutrition in countries where beans, which are particularly likely to infection by strains of varying effectiveness, are an important protein source. Research focused on the identification of both competitive and highly efficient nitrogen-fixing strains has been limited because so far, the only way to screen rhizobial strains has been to isolate individual strains and compare them one at a time in large-scale plant growth assays.

The principal aim of this project is to engineer tools to quickly assess competitive and effective rhizobial strains in a large population of native soil rhizobia allowing us to compare multiple strains at once and thus to carry out a high-throughput screening for competitive and efficient strains.

We have developed novel reporter plasmids, which include the biomarkers *gusA* or *celB* under the control of a synthetic *nifH* promoter. These reporter plasmids were constructed by an integrated high-throughput strategy using the latest 'Golden Gate' cloning technique [3].

We transferred these plasmids to *Rhizobium leguminosarum* bv. viciae *3841* and followed the competition methodology from Sánchez-Cañizares & Palacios (2013). The competition assays carried out with our reporter plasmids show that they do not affect the competitiveness of *Rhizobium leguminosarum* bv. viciae 3841. Thus, our results demonstrate that by using synthetic biology we can produce a suitable bioreporter that will allow us to carry out a high-throughput screening for competitive and efficient strains. In order to achieve this, the next step in this project will be the addition of barcodes to enable rapid identification of successful strains, allowing us to compare multiple strains at once.

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POSTER 4-5 /LIGHTNING TALK/

Plant as an Evolutionary Driver of Symbiotic Microbiome

Anna Igolkina, O. Onishchuk, O. Kurchak, E. Chirak, E. Andronov, N. Provorov

ARRIAM, St-Petersburg, Russia

The main hypothesis of the current project is the strong plant influence on soil microbiome structure. Testing it we applied the modern molecular methods and multilevel bioinformatic analysis. Our project was aimed to detect plant-microbe parallel evolution in nitrogen-fixing *Rhizobium*-legume symbiotic system, to be specific, between its two components: rhizobial symbiotic gene *nodA* and plant receptor gene *nfr5*. These genes play an essential role in symbiotic partners recognition.

Preparing the initial data we massively sampled three wild growing (from one compact territory) legume plants (*Vicia, Lathyrus, Trifolium*): plant tissue, root nodules and corresponding rhizosphere soil samples. Finally, for each particular plant we created three DNA pools: from plant, nodules and soil. We used degenerate primers to construct amplicon libraries. These libraries were analyzed with pyrosequencing (*nodA*) and Sanger sequencing (*nfr5*).

Using high-throughput data we developed the original computational pipeline for detection of plant-driven variability in soil microbiome. It consisted of several hierarchical steps from phylogenetic analysis to parallel diversity analysis of both bacterial and plant genes. We first performed OTU-picking for total *nodA* gene sequences and detected the frequency-dependent selection during bacterial transition into a host plant. Next, the diversity analysis on haplotype and nucleotide levels showed the increased diversity of *nodA* alleles in root nodule population compared to the soil one. Haplotype frequencies shift in root-nodule population reveals symbiosis-specific selection patterns how a plant chooses specific bacteria.

On the next step we studied the source of the variability in root nodule population and developed the modification of dn/ds statistics adapted to our data. We found high level of both synonymous and nonsynonymous substitutions in root-nodule population and significantly increased value of dn/ds statistics. These results characterise the type of natural selection acting on the root-nodule population and reveal positions in nodA gene under this selection.

Finally, we compared nucleotide diversity values between *nfr5* gene in plants and *nodA* genein root-nodule populations and found these values linearly dependent.

All obtained results demonstrated quite clear and spectacular picture of co-evolutionary forces in symbiotic systems and especially diversity analysis showed the guide role of a plant in evolution of soil microbiome.

This work is supported by RSF grant 14-26-00094.

POSTER 4-6 /LIGHTNING TALK/

Effect of Phosphate Solubilization on Nitrogen Fixation in Clover

Kritarth Seth[1], Derrick Moot[1], Maureen O'Callaghan[2], Hayley Ridgway[1]

[1] *Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln, New Zealand*
[2] *AgResearch Ltd, Lincoln Research Centre, Christchurch, New Zealand*

The ability of legumes to fix nitrogen (biological nitrogen fixation; BNF) in symbiosis with rhizobia is limited by soil P availability. This is because BNF requires 5 - 10% more energy than assimilation of the same amount of soil N (1). Thus, the balance between N and P is critical for effective BNF.

Phosphorus, being highly reactive, is the most immobile element in soil with only 5 to 30% of fertilizer inputs utilized by plants. The remainder complexes with metal ions and accumulates in soil as insoluble, unusable deposits. Microbial phosphate solubilization (PS) via the secretion of organics acids has been reported to make more P available for plant uptake. Many bacterial species can solubilise P *in vitro* but most have failed to do so *in planta*. This means there is no large scale bacterial commercial product for PS. The objective of this study was to screen nodule inhabitants of clover for PS and to study the effects of higher P availability on BNF.

A total of 2220 nodule inhabiting bacteria were collected from four sites with contrasting long term P fertilization histories, using white (*Trifolium repens*) and subterranean (*T. subterraneum*) clovers as bait plants. Isolates were screened for their ability to solubilise P *in vitro* using media containing highly insoluble hydroxyapatite (HA) as the sole source of P. Only 79 (3.6%) of bacteria were found to solubilise P. More isolates originating from subterranean than white clover nodules could solubilise P. This suggests an active host recruitment ($p<0.05$) of phosphate solubilizing bacteria (PSB). A higher ($p<0.05$) proportion of PSB was also recovered from plants grown in soil that had received high levels of P, suggesting adaptation of bacteria to solubilise P in these soils.

Only 25 of the 79 PSB were *Rhizobium spp.*, despite all originating from within clover nodules. Of these, 11 isolates (*R. leguminosarum* bv. *trifolii*) were able to nodulate white clover and fix nitrogen. When white clover plants were supplemented with minimum nitrogen and HA as the only P source, all 11 isolates increased plant growth in comparison with the uninoculated control. When used to inoculate white clover, the best isolates produced a 73% increase in dry weight compared with our standard commercial inoculant *R. leguminosarum* bv. *trifolii* TA1.

The best isolates found in this study increased plant growth by a combined effect from fixing N and solubilising P. These dual function inoculants provide the potential for sustainable P utilization and improved nitrogen fixation.

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POSTER 4-7 /LIGHTNING TALK/

Quorum Sensing Controls Phenotypic Heterogeneous Expression of the Autoinducer Synthase Gene *trai* via Copy Number Control of pNGR234*a* in the Plant Symbiont *S. Fredii* NGR234

Katrin Petersen[1], J. Grote[1], D. Krysciak[1], C. Schmeisser[1], K. U. Förstner[2], H. Krishnan[3], W. R. Streit[1]

[1] *Biozentrum Klein Flottbek, Abteilung für Mikrobiologie und Biotechnologie, Universität Hamburg, Hamburg, Germany*
[2] *Core Unit Systems Medicine, Universität Würzburg, Würzburg, Germany*
[3] *Plant Genetics Research Unit, United States Department of Agriculture-Agricultural Research Service, University of Missouri, Columbia, Missouri, United States*

Sinorhizobium Fredii NGR234 is a plant symbiont which is able to form nitrogen-fixing nodules in over 120 plant genera. Its genome encodes for two autoinducer (AI) systems in which the TraI system is localized on the symbiotic plasmid pNGR234*a* that is in part highly similar to the *A. tumefaciens* Ti plasmid.

qPCR analyses of copy numbers of pNGR234a of single AI deletion mutants *S. fredii* NGR234 Δ*traI* and Δ*ngrI* and the corresponding double AI-synthase mutant indicate the partial or complete lack of AI molecules affects the copy number of the pNGR234*a* replicon. In general, the copy number was altered in response to mutations affecting the AI regulons and by the addition of external AI molecules. The increased copy number observed in the absence of any AI basically eliminated the previously described phenotypic heterogeneous expression of the *traI* gene4 and caused a low level expression of virtually all genes on pNGR234*a*. RNA-seq data in the background of a Δ*traI*, a ⊿*ngrI* and a Δ*traI*⊿*ngrI* double mutant indicate that the copy number control of pNGR234*a* and the phenotypic heterogeneity are linked with to two novel ORFs identified on the symbiotic replicon. The two ORFs encode for a 51 aa and a 143 aa protein located in the region of the oriV of pNGR234*a*. We have designated these ORFs *repX* and *repA0* and both have previously not been reported. Both proteins are unique to broad host range rhizobia and not present in *A. tumefaciens* or related bacteria affiliated with the rhizobiales. Overexpression of *repA0 in NGR234 wt cells* increases the copy number of pNGR234*a*. Therefore we speculate that both ORFs are part of NGR's unique tool box that enables it to nodulate such a wide spectrum of legume plants.

References:

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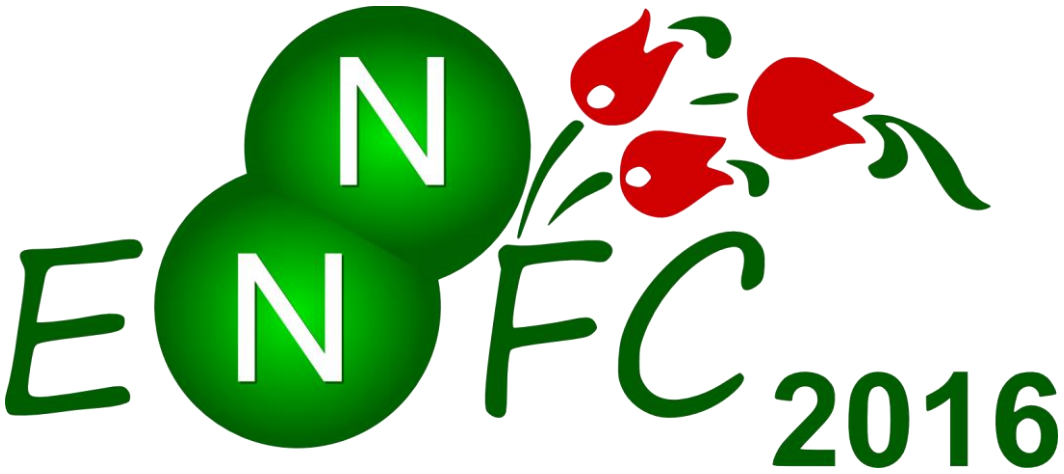

PLENARY SESSION 5A

Functioning of the N-fixing Symbioses /Bacteria/

Chair: Jose Palacios

Metabolic Transitions of Rhizobia

Philip Poole[1], Vinoy Ramachandran[1], Isabel Webb[1], Jason Terpolilli[2]

[1] *Department of Plant Sciences, University of Oxford, Oxford, United Kingdom*
[2] *Centre for Rhizobium Studies, Murdoch University, Perth, Australia*

Colonization by bacteria of the zone surrounding plant roots (rhizosphere) is crucial to plant productivity, with plants secreting 10-30% of total photosynthate to engineer the rhizosphere to their advantage. Microarray and metabolic analysis has been used to dissect the composition of the pea root secretome and map the transcriptional response of bacterial to secreted metabolites. Most recently this work has led to identification of the master regulator of attachment of *Rhizobium leguminosarum* to pea roots. During infection of legumes the metabolic repertoire of rhizobia is dramatically restricted with a dramatic reduction in metabolic diversity in mature bacteroids. The role of the FixABCX proteins in electron allocation to nitrogenase will be considered and the metabolic changes that this leads to will be considered. Finally, the metabolism of bacteroids from indeterminate pea nodules will be copared to determinate bean nodules.

Plasticity of α-rhizobial Genomes: A Cell Biological Perspective

J. Döhlemann, M. Wagner, M .Brennecke, B. Frage, Anke Becker

LOEWE Center for Synthetic Microbiology and Faculty of Biology, Philipps-Universität Marburg, Germany

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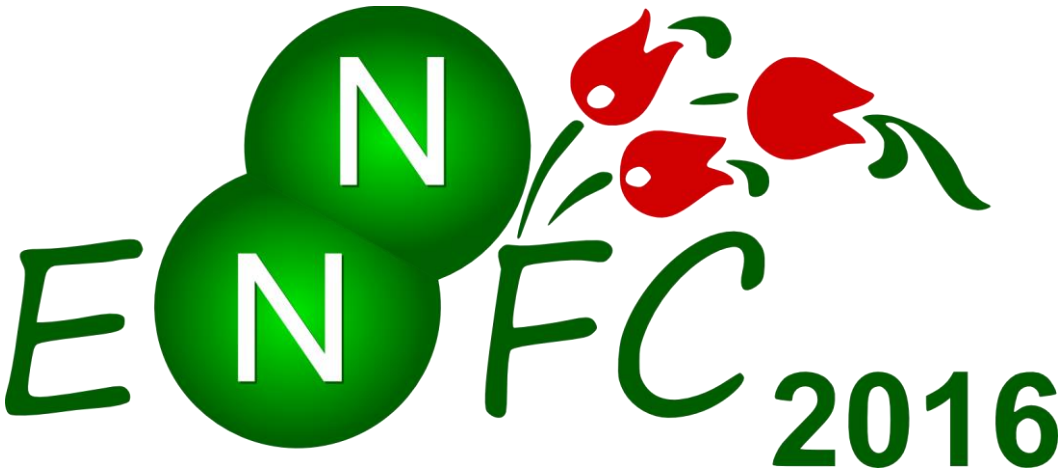
About 10% of sequenced bacterial species maintain multipartite genomes. The precise spatiotemporal coordination of genome replication and segregation with cell growth and division is vital for proliferation of these bacteria. Multipartite genomes are particularly prevalent in plant-symbiotic α-rhizobia. In this group of bacteria, they usually comprise one chromosome and two to six RepABC-family plasmids, which are characterized by the combined replication and partitioning *repABC* locus. While RepC most likely acts as the replication initiator protein at the origin of replication, RepA, RepB and the partitioning sites are required for vertical transmission of the megaplasmids to the daughter cells. Natural populations of α-rhizobial species show extensive variations at genetic level, with the highest variation observed on the RepABC-type plasmids. Naturally occurring replicon fusions resulting from homologous recombination events have been observed in various α-rhizobial species.

Sinorhizobium meliloti possesses a tripartite genome composed of one main chromosome (3.65 Mb) and the RepABC-type megaplasmids pSymA (1.35 Mb) and pSymB (1.68 Mb). We observed that duplication of the chromosomal and megaplasmid origins of replication is spatially and temporally uncoupled and occurs only once per cell cycle. Origin partitioning follows a strict temporal order, commencing with the chromosome, and followed by pSymA and then by pSymB. This suggests coordination of replication and segregation of α-rhizobial chromosomes and RepABC-family plasmids.

We have established a genetic platform for induction of site-directed genome rearrangements including deletions, inversions, replicon fusions and establishment of additional *repABC*-based plasmids. This toolbox has been applied to study robustness of the α-rhizobial cell cycle against genome rearrangements.



PLENARY SESSION 6

Biological Nitrogen Fixation in Non-Legume Environments

Chair: Pepe Palacios

Diatom-N_2 Fixing Symbioses: Making the Most in a Nutrient Deplete Open Ocean

Rachel A. Foster, Andrea Caputo, Andreas Novotny, Marcus Stenegren

Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm Sweden

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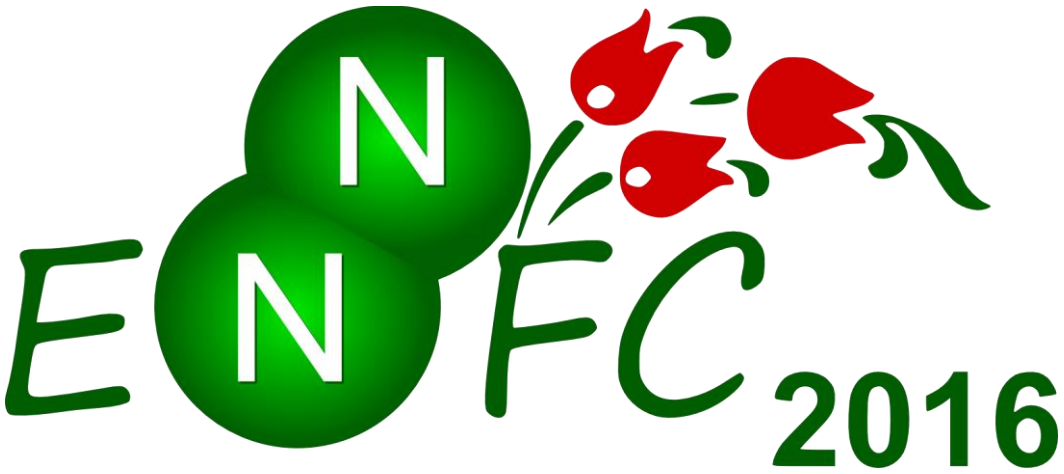
Some of the most enigmatic components of the marine plankton are the microalgal groups which carry intimately associated N_2-fixing cyanobacteria as symbionts. The initial discovery for many of these planktonic partnerships dates back to well over a 100 years ago, yet we still know precious little about the partners and their role for one another. There are several different diatom genera that take up N_2 fixing heterocystous cyanobacteria and one host diatom genus associates with unicellular cyanobacteria. All partnerships are considered highly specific and quite common in low nutrient areas of the open ocean and the symbiont's primary function is the provision of fixed N. What factor(s) influence the distribution of the symbioses is largely unknown. The selectivity and host specificity is largely unresolved and of interest. The location of the symbionts (e.g. internal or external) and ultrastructure of the hosts and mechanism of nutrient exchange is largely unstudied, including the nature of nutrient transfer.

Using a variety of methodologies including single gene PCR and sequencing, qPCR, confocal microscopy, stable isotope amendment experiments and nano-meter scale secondary ion mass spectrometry, we have begun to unravel several intriguing aspects for these planktonic partnerships, including the host diversity, symbiont location, the nature of exchanges and dependency between the partners, and the potential drivers which influence their distribution in the global ocean.



PLENARY SESSION 5B

Functioning of the N-fixing Symbioses /Plants/

Chair: Péter Kaló

Deconstructing Symbiosis: Loss-of-function Mutations Reveal Key Genes for Symbiotic Nitrogen Fixation in *Medicago truncatula*

S. Sinharoy[1,2], C. Pislariu[1,3], I. Kryvoruchko[1,4], I.Torres-Jerez[1], S. Zhang[1], P. Routray[5], D. Sosso[6], C. Liu[7], A. Breakspear[7], D. Guan[7], S. Shailes[7], P-M. Delaux[8], J. Nakashima[1], J. Wen[1], G. Oldroyd[7], J-M. Ane[8], V. Benedito[9], J. Murray[7], W. Frommer[6], D. Roberts[5], Michael Udvardi[1]

[1] *Samuel Roberts Noble Foundation, United States*
[2] *University of Calcutta, India*
[3] *Texas A&M International University, United States*
[4] *Kafkas University, Turkey*
[5] *University of Tennessee, United States*
[6] *Carnegie Institution of Science, United States*
[7] *John Innes Centre, United Kingdom*
[8] *University of Wisconsin, United States*
[9] *West Virginia University, United States*

We screened a *Medicago truncatula Tnt1* mutant population to identify mutants defective in symbiotic nitrogen fixation (SNF). *Tnt1* insertions in two genes, *MtCBS1* and *MtNPD1*, were found to affect rhizobial colonization and/or persistence in nodules and SNF. Reverse-genetic screening of the *Tnt1* mutant population identified insertions in two transporter genes of interest, *MtMATE3* and MtSWEET11, one of which (MtMATE3) was required for effective SNF.

MtCBS1 encodes a protein of unknown function with a putative membrane-localized DUF21 domain and a cystathionine-beta-synthase (CBS) domain. *cbs1* mutants exhibited defective infection threads and produced small nodules with reduced numbers of symbiosomes. *MtCBS1* was expressed in infected root hair cells, developing nodules, and in the invasion zone of mature nodules. An MtCBS1-GFP fusion protein localized to the infection thread and symbiosomes. Related proteins have been implicated in plant cell wall maturation, indicating a potential role for CBS1 in the formation of the infection thread wall (Sinharoy et al. (2016) Plant Physiol. 170, 2204-17).

MtNPD1 encodes another protein of unknown function, with a PLAT (Polycystin-1, Lipoxygenase, Alpha-Toxin) domain. Although early stages of nodule development and *S. meliloti* colonization appeared to be normal in *npd1* mutants, nodules ceased to grow after a few days, resulting in small, ineffective nodules. Rhizobia that colonized developing *npd1* nodules were degraded prematurely. Expression of *MtNPD1* accompanied invading rhizobia in the infection zone and into the nodule interzone. *MtNPD1* belongs to a cluster of 5 nodule-specific single PLAT domain-encoding genes, which apparently have non-redundant functions.

MtSWEET11 was expressed in infected root hair cells, and in the meristem, invasion zone, and vasculature of nodules. When expressed in mammalian cells, MtSWEET11 transported sucrose but not glucose. Expression of MtSWEET11-GFP in nodules resulted in green fluorescence associated with the plasma membrane of uninfected cells and infection thread and symbiosome

membranes of infected cells. Two *sweet11* mutants were uncompromised in SNF. Therefore, MtSWEET11 likely shares a role in nodule sucrose transport with other transporters (Kryvoruchko et al. (2016) Plant Physiol. 171, 554-65).

MtMATE3 transcript levels increased 1000-fold during nodule development. Heterologous expression and transport assays in *Xenopus* oocytes indicated that MtMATE3 is a citrate transporter stimulated by Fe^{3+}. *mate3* mutants were impaired in nodule development and nitrogen fixation. MtMATE3 may play a role in nodule iron homeostasis.

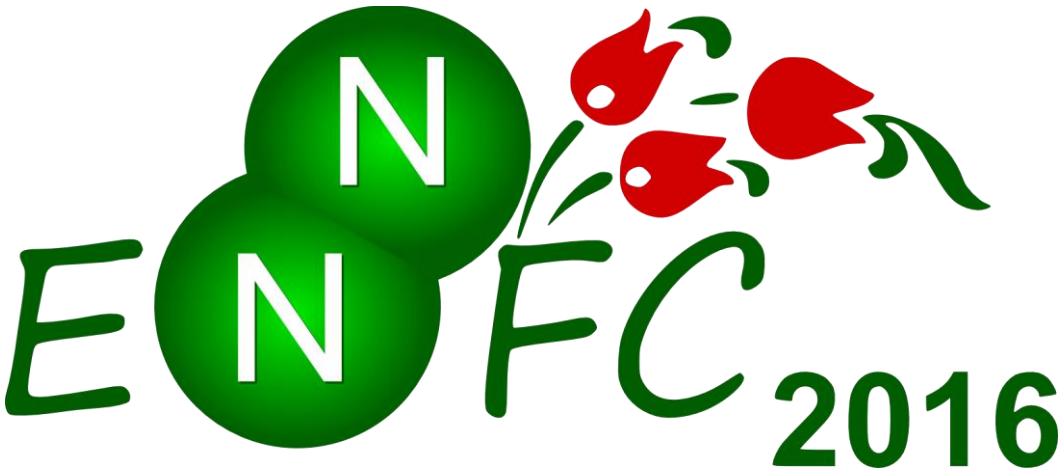
A Band of Misfits: Role of Unexpected Proteins in the Plant Symbiotic Signaling Pathway

Muthusubramanian Venkateshwaran[1,a], Audrey Wiley-Kalil[1], Dhileepkumar Jayaraman[1], Mari Banba[2], Andreas Binder[3], Shane Bernard[4], Mireille Chabaud[5], Junko Maeda[4], Kari Forshey[1], Désirée Den Os[1], Andrea Genre[6], Marisa Otegui[7], David G. Barker[5], Haruko Imaizumi-Anraku[2], Martin Parniske[3] and Jean-Michel Ané[1,4]

[1] *Department of Agronomy, University of Wisconsin, Madison, United States*
[2] *Division of Plant Sciences, National Institute of Agrobiological Sciences (NIAS), Tsukuba, Ibaraki, Japan*
[3] *Ludwig-Maximilians-Universität München, Faculty of Biology, Genetics, Martinsried, Germany*
[4] *Department of Bacteriology, University of Wisconsin, Madison, United States*
[5] *Laboratory of Plant-Microbe Interactions, Institut National de Recherche Agronomique (UMR 441), Centre Nationale de Recherche Scientifique (UMR 2594), Castanet-Tolosan, France*
[6] *Department of Life Sciences and Systems Biology, University of Torino, Italy*
[7] *Department of Botany, University of Wisconsin, Madison, United States*

[a] *Current address: School of Agriculture, University of Wisconsin, Platteville, United States*

Rhizobia and arbuscular mycorrhizal fungi produce lipo-chito-oligosaccharidic signals that are perceived by legume hosts at the plasma membrane level. These signals are then transduced to the nucleus where they trigger oscillations of the nuclear calcium concentration (calcium spiking) that, in turn, regulates symbiotic gene expression. Genetic and protein interaction studies identified some relatively expected components in such a signaling pathway: receptor-like kinases at the plasma membrane that perceive the microbial signals, ion channels and ion pumps on the nuclear envelope that allow calcium spiking, and nuclear transcription factors that regulate gene expression. We will present new data on more unexpected members of this signaling pathway. (1) An HMGR (3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase) that interacts with and is regulated by the symbiotic receptor kinases. In particular, we will show that the activity of this HMGR is not only necessary but also sufficient to trigger calcium spiking. (2) Nucleoporins of the NUP107-160 sub-complex that have been identified through forward genetic approaches. We will show that these nucleoporins are required for the proper localization of symbiotic ion channels to the inner nuclear membrane. Finally, we will compare results between two legumes, *Medicago truncatula* and *Lotus japonicus*, and show the value of comparing carefully these two genetic systems.



PLENARY SESSION 7

Free-living Nitrogen Fixation

Chair: Péter Kaló

Intercellular Communication in the Diazotrophic Filament of Heterocyst-forming Cyanobacteria

Enrique Flores

Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC and Universidad de Sevilla, Seville, Spain

Filamentous, heterocyst-forming cyanobacteria are multicellular organisms in which, under nitrogen-limiting conditions, growth requires the activity of two interdependent cell types: the vegetative cells that carry out oxygenic photosynthesis and the heterocysts that perform nitrogen fixation (1). Heterocysts differentiate from vegetative cells in a process that involves a complex program of gene expression and the intercellular transfer of regulators. In the diazotrophic filament, vegetative cells provide heterocysts with reduced carbon (mainly in the form of sucrose) and heterocysts provide the vegetative cells with fixed nitrogen. The heterocysts conspicuously accumulate cyanophycin, a polymer made of aspartate and arginine that is a dynamic reservoir of nitrogen. A cyanophycin degradation intermediate, β-aspartyl-arginine, is transferred as a nitrogen vehicle from heterocysts to vegetative cells (2). The cyanobacteria bear a Gram-negative type of cell envelope, and the cyanobacterial filament consists of individual cells surrounded by their peptidoglycan layers but enclosed in a continuous outer membrane that defines a continuous periplasm. The cells in the filament appear to be connected by septal junctions that traverse the septal peptidoglycan through perforations (termed nanopores) that can be visualized by electron microscopy. Intercellular communication can be probed with fluorescent tracers including calcein, 5-carboxyfluorescein and the sucrose analogue esculin. Filament fragmentation mutants have led to the identification SepJ, FraC and FraD, which are integral membrane proteins that are located at the cell poles in the intercellular septa of the filament (3). Mutants lacking these proteins show significantly decreased numbers of nanopores and are impaired in the intercellular transfer of tracers, identifying SepJ, FraC and FraD as possible components of septal junctions (4). However, *sepJ* and *fraC-fraD* mutants are distinct suggesting that two types of septal junctions exist, one related to SepJ and another related to FraCD (3, 4). The septal junctions, which can be considered analogous to metazoan gap junctions, appear to mediate intercellular transfer of nutrients and regulators in the diazotrophic filament of heterocyst-forming cyanobacteria (5).

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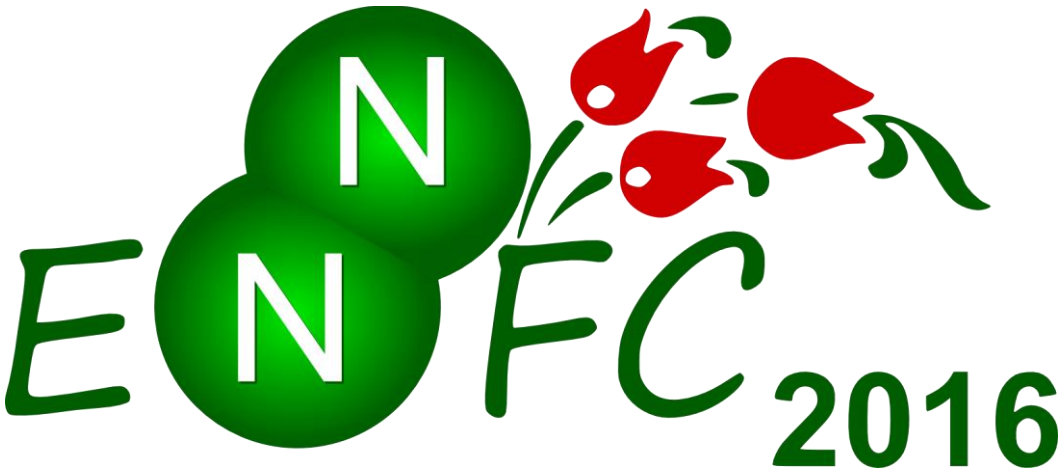

PARALLEL SESSION 5A

Functioning of the N-fixing Symbioses /Bacteria/

Chairs: Sharon Long, Emanuele Biondi

A Hypothesis for the Acquisition and Evolution of Peptides Controlling Differentiation of Nitrogen Fixing Rhizobia in Legume Nodules

J. Allan Downie[1] and Eva Kondorosi[2]

[1] *John Innes Centre, Colney, Norwich, United Kingdom*
[2] *Institute of Biochemistry, Biological Research Centre HAS, Szeged, Hungary*

There are marked differences between the fates of rhizobia within the nodules of legumes such as *Medicago truncatula* and pea (belonging to the 'IRLC' Invert-Repeat Lacking Clade) compared with those in legumes such as *Lotus japonicus* and soybean not in the IRLC clade. Within nodules of IRLC legumes, the rhizobia become terminally differentiated and this is caused by genes encoding small antimicrobial-like peptides. In *M. truncatula*, there are over 800 genes encoding such peptides that are specifically expressed in nodules and targeted to bacteroids by signal peptides and a plant-made secretion complex. In contrast, legumes such as *L. japonicus* and soybean lack the genes encoding such antimicrobial-like peptides. Since rhizobia can form efficient nitrogen-fixing symbioses with non IRLC legumes it is evident that these peptides are not required *per se* for nitrogen fixation by rhizobia. However in *M. truncatula* mutations affecting at least some of these peptides block nitrogen fixation. This implies an interdependence of peptides, such that in the absence of some peptides, others may interfere with nitrogen fixation.

The numbers of such peptides varies greatly in different IRLC legumes, with significant differences between phylogenetically close species. This implies rapid acquisition and evolution of these peptides. However this raises the conundrum as to why such peptides should have evolved in IRLC legumes such as *M. truncatula* and pea, but not in non-IRLC legumes such as *L. japonicus* and soybean.

We will propose a model in which nodule-specific antimicrobial peptides may have been selected to control development and/or metabolism of rhizobia. This model could explain why many peptides evolved in some legumes. We will also propose reasons, based on nodule infection structures, why some legumes are more likely to control rhizobial differentiation by nodule-specific peptide expression.

Bacterial Cell Cycle and Bacteroid Differentiation are Linked in *Sinorhizobium meliloti*

Elisa Giuntini, Francesco Pini*, Emanuele G. Biondi

Aix-Marseille University, CNRS UMR 7283 LCB, 13009 Marseille, France

** Present address: Department of Plant Science, University of Oxford, United Kingdom*

In all domains of life, proper regulation of the cell cycle is critical to coordinate genome replication, segregation and cell division. In some groups of bacteria, *e.g. Alphaproteobacteria,* tight regulation of the cell cycle is also necessary for the morphological and functional differentiation of cells. During the symbiosis in *Medicago* species, the alphaproteobacterium *Sinorhizobium meliloti* undergoes an elaborate cellular differentiation within host root cells. This differentiation results in massive amplification of the genome, cell branching and elongation, and loss of reproductive capacity (1). In the closely related alphaproteobacterium *Caulobacter crescentus*, cellular differentiation is tightly linked to the cell cycle via the activity of the master regulator CtrA, and recent research in *S. meliloti* suggested that CtrA might also be key to cellular differentiation during symbiosis (2). Depletion of CtrA causes cell elongation, branching and genome amplification, similar to that observed in nitrogen-fixing bacteroids (3). We showed that the cell cycle regulated proteolytic degradation of CtrA is essential in *S. meliloti* and all mutants of the CtrA degradosome are impaired in the differentiation process. As CtrA must be absent in mature bacteroids allowing a proper nitogen fixation we hypothesized that CtrA proteolysis may play a crucial role in bacteroid development and possibly triggered by NCR plant peptides. Our findings provide valuable insight into how highly conserved genetic networks can evolve, possibly allowing complex differentiation programs such as the bacteroid differentiation in *S. meliloti.*

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Development of a Permissive Platform for Identification of the Minimal Rhizobial Symbiotic Genome and Forward Genetic Analyses

George C. diCenzo, Maryam Zamani, Branislava Milunovic, Turlough M. Finan

Department of Biology, McMaster University, Hamilton, Ontario, Canada

The *Sinorhizobium meliloti* – *Medicago* symbiosis is a well-studied model system whose analysis is enriched by the wealth of genetic techniques and genomic resources available for *S. meliloti*. The genome of *S. meliloti* Rm2011 consists of a 3.7 Mb chromosome, and the 1.4 Mb pSymA and 1.7 Mb pSymB replicons, with the great majority of horizontally acquired symbiotic genes present on pSymA and pSymB. Here, we detail the steps we have taken to construct a permissive background *S. meliloti* strain for the end goals of identifying the necessary and sufficient *S. meliloti* symbiotic genome and to enable subsequent forward genetic analyses of this process. We identified a 69 kilobase region on pSymB carrying genes essential for viability (*engA* and an arginine tRNA) and symbiosis (*bacA*), whose presence on pSymB is the result of a translocation from the chromosome following the divergence from *Sinorhizobium Fredii* NGR234 (1,2). We transferred the entire 69 kb region from the *S. fredii* NGR234 chromosome into the ancestral location of the *S. meliloti* Rm2011 chromosome, and doing so facilitated the construction of a *S. meliloti* strain lacking the entire pSymA and pSymB replicons (termed ΔpSymAB), a 45% genome reduction (2,3). The ΔpSymAB strain was Nod-Fix- with *Medicago sativa*, and re-introduction of both pSymA and pSymB fully restored symbiotic capabilities (2). These results therefore illustrate ΔpSymAB as a permissive background for forward genetics of symbiosis and testing of the sufficiency of a putative minimal symbiotic gene set. As a first step towards elucidating the minimal symbiotic genome, we generated quantitative *M. sativa* shoot dry weight data for plants individually inoculated with strains from a pSymA/pSymB deletion library that cumulatively removes greater than 95% and 2,500 genes of these replicons (2,4). Several regions were identified that contributed to the effectiveness of the symbiosis, but only 4 regions that accounted for a total of less than 12% of pSymA and pSymB were identified as carrying essential symbiotic genes (2). These regions will serve as an initial target of the necessary and sufficient horizontally transferred symbiotic genes, and we have begun combining the Fix+ deletions to identify whether additional, unidentified functionally redundant genes pairs required for symbiosis are located on these replicons. Furthermore, the ability of the *S. fredii* NGR234 *bacA* gene integrated into the *S. meliloti* Rm2011 chromosome as part of the 69 kb region to complement the loss of the pSymB encoded *bacA* gene will be discussed.

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Characterization of *Sinorhizobium meliloti* Mutants with Increased Resistance towards NCR Peptides

Etelka Kovács, Éva Kondorosi, Attila Kereszt

Institute of Biochemistry, Biological Research Centre HAS, Szeged, Hungary

Since the occurrence of antibiotic-resistant superbugs resulting from the widespread use of conventional antibiotics is multiplying, numerous studies have focused on the use of antimicrobial peptides (AMPs) as therapeutic agents.

A large group of AMP-like molecules with up to 600 different members is the nodule-specific cysteine-rich (NCR) peptide family, which is produced almost exclusively in the infected cells of the nitrogen-fixing nodules of *Medicago truncatula*. NCR peptides contain a relatively conserved signal peptide and a highly diverse mature peptide composed of 30–50 amino acids with conserved positions of four or six cysteines.

Cationic NCR peptides have been found to have antimicrobial activity, killing *Sinorhizobium meliloti* (and other Gram-negative and Gram-positive bacteria as well as fungi) when applied at high concentration. To counteract the antimicrobial activity of the NCR peptides, rhizobia requires the BacA protein. In the absence of this protein, the bacteroids do not differentiate, rather they are immediately killed by the NCR peptides in the nodule as soon as they are released in the symbiosomes.

We evolved increased tolerance against a peptide in the wild-type and the *bacA* mutant strains of *S. meliloti* 1021 by increasing gradually the concentration of NCR335 in the growth medium. The lines became tolerant not only towards the NCR335 peptide but also against other cationic peptides such as NCR247 and indolicidin isolated from bovine neutrophils. Genome sequencing of the peptid-tolerant lines revealed that they accumulated 5-10 mutations during their evolution. To test the effect of the individuals mutations on peptid sensitivity both in free-living state and *in planta*, they have been transferred into the genome of parental strains as well as the mutant genes cloned in a plasmid have been expressed from the *bacA* promoter.

The mutations that can render the cells to tolerate 2-3 times higher concentration of NCR peptides than the wild type strain and can rescue the symbioic phenotype of the bacA mutant will be presented and discussed.

POSTER 5A-1 /LIGHTNING TALK/

sRNA-mediated Regulation of the Cell Cycle Master Regulator CtrA in *Sinorhizobium meliloti*

Marta Robledo[1,2], J. P. Schlüter[1], L. O. Loehr[1], U. Linne[3], S. Albaum[4], J. I. Jiménez-Zurdo[2], A. Becker[1]

[1] *LOEWE Center for Synthetic Microbiology and Faculty of Biology, Philipps-Universität Marburg, Germany*
[2] *Grupo de Ecología Genética de la Rizosfera, Estación Experimental del Zaidín (CSIC), Granada, Spain*
[3] *Department of Chemistry, Philipps-Universität Marburg, Germany*
[4] *Center for Biotechnology CeBiTec, Universität Bielefeld, Germany*

Small untranslated RNAs (sRNAs) are widespread post-transcriptional regulators that modulate fundamental aspects of bacterial physiology in response to environmental conditions. Regulation of the cell cycle to retard chromosome replication, segregation and/or cell division under stress conditions is crucial for bacteria. In α-proteobacteria, tight control of the cell cycle is also necessary to achieve genome replication only-once per generation, as observed in *Caulobacter crescentus,* or to accomplish cellular differentiation within legume host root cells, like most symbiotic α-rhizobia. Coordination of DNA replication and cytokinesis in model α-proteobacteria is ensured by cyclically changing concentrations of the conserved transcriptional regulators DnaA, GcrA and CtrA[1]. DnaA and GcrA control transcription of genes involved in early cell cycle events, whereas the essential master regulator CtrA directly controls genes primarily involved in late cell cycle events, motility and asymmetry[2]. We have recently reported a conserved sRNA modulating expression of *dnaA* and *gcrA* under stress conditions in α-rhizobia[3]. However, post-transcriptional mechanisms controlling cell division are still poorly explored.

The *S. meliloti* trans-encoded sRNA GspR (Growth Stop Phenotype RNA) was selected from a phenotypic screening of sRNA overexpression strains. The growth arrest phenotype was observed in all *Sinorhizobium* species carrying GspR homologs. Northern blot analysis confirmed production of this sRNA under different growth and stress conditions. Its predicted secondary structure contains three stem-loops (SL). CopraRNA-based predictions of mRNA targets of SL1 and SL3 showed an enrichment for cell division-related genes. Transcriptome and proteome analyses identified several genes which were differentially expressed dependent on GspR. Both approaches identified *ctrA*, which was less abundant in the *gspR* overexpression strain, along with proteins linked to cell motility and cellular metabolism. GspR-dependent altered translation of *ctrA* mediated by mRNA base-pairing with SL1 was experimentally confirmed by an eGFP-based reporter system and compensatory changes in sRNA and mRNA.

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POSTER 5A-2 /LIGHTNING TALK/

Inactivation of PhaR Involved in Poly-beta-hydroxybutyrate Accumulation in *Bradyrhizobium japonicum* USDA110 and its Pleiotropic Effects

Shogo Nishihata[1], Kosei Tanaka[2], Shinji Takanaka[1,2] and Ken-ichi Yoshida[2, 3]

[1] *Department of Agrobioscience, Kobe University, Kobe, Japan*
[2] *Organization of Advanced Science and Technology, Kobe University, Kobe, Japan*
[3] *Department of Science, Technology and Innovation, Kobe University, Kobe, Japan*

Bradyrhizobium japonicum USDA110 accumulates poly-beta-hydroxybutyrate (PHB) in the cells grown with an excess of carbon sources. Previously we demonstrated that PhaP proteins (phasins) stabilizing PHB granules played important roles in PHB accumulation in USDA110 (1). PhaR repressor was suggested to regulate expression of the *phaP* genes but remained to be elucidated experimentally. In this study, we created and analyzed a *pha*R-deficient mutant strain to investigate its changes in various phenotypes, including bacterial growth, PHB accumulation, and symbiotic nitrogen fixation. In addition, PhaR was produced and purified in *Escherichia coli* to analyze its DNA binding in vitro.

When the *pha*R-deficient mutant was cultured in non-PHB-accumulating conditions, among the genes involved in PHB accumulation, transcription levels of *phaP1*, *phaP4*, and *phbZ1* were elevated significantly, indicating that PhaR could be involved in transcriptional repression of the two phasins, PhaP1 and PhaP4, as well as the PHB degrading enzyme, PhbZ1. Indeed, in vitro experiments, including gel mobility shift and DNase I foot print analyses, revealed that PhaR specifically bound to the promoter regions of these genes, identifying its function as transcriptional repressor.

When cultured in PHB-accumulating conditions, growth of *pha*R-deficient mutant was impaired, and its intracellular PHB accumulation was reduced more pronouncedly than in the *phaP4*-deficient mutant. On the other hand, it secreted twice more exopolysaccharides (EPS) than both the parental and the *phaP4*-deficient strains. The results suggest that elevated expression of the two phasins and/or the PHB degrading enzyme might cause the less efficient PHB accumulation. Moreover, inactivation of *pha*R might switch the cellular metabolism from PHB accumulation to EPS secretion, and thus caused changes in the metabolism might restrict the cell growth. Unexpectedly, the *pha*R-deficient mutant showed somehow elevated resistance to thermal stresses, although non-PHB-accumulating mutants of other microorganisms reportedly exhibited less tolerance to various stresses.

When soybean was infected with *pha*R-deficient mutant, nodules were formed almost similarly as infected with the parental strain, and normal growth of soybean plant was observed without supplemental nitrogen sources. The results suggest that neither the less efficient PHB accumulation nor the enhanced EPS secretion might affect the establishment of symbiosis and nitrogen fixation. Since PHB may serve as energy storage compound in bacteroides, we are currently investigating effects of the *pha*R inactivation on efficiency in bacterial outgrowth from matured nodules.

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POSTER 5A-3 /LIGHTNING TALK/

Insertion Sequencing in *Rhizobium leguminosarum* bv. viciae 3841

Rachel M. Wheatley, Vinoy K. Ramachandran, Philip S. Poole

Department of Plant Sciences, University of Oxford, Oxford, United Kingdom

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Rhizobium leguminosarum bv. *viciae 3841* belongs to the symbiovar *viciae* that establishes nitrogen-fixing symbiosis with *Viciae* legumes such as agriculturally important pea (*Pisum sativum*) and vetch (*Vicia craccca*). Microarray transcriptomic and bioinformatics studies carried out in *Rhizobium* have suggested many gene functions but they do not necessarily identify those important for growth in a particular environment or those required for symbiosis (1-2). Insertion sequencing (INSeq) can be used to study gene fitness at the genome scale. It involves subjecting large libraries of Mariner transposon insertion mutants to high-throughput sequencing to assess how mutants are altered in growth and survival (3).

InSeq has been coupled with a four-state Hidden Markov Model (HMM) for analysis (4) which classifies genes as growth-essential, -defective, neutral or -advantaged.

INSeq has been used to investigate a variety of biological questions in Rlv3841; including pea root attachment, growth in the pea rhizosphere, growth on a C4 carboxylic acid carbon source, and for growth at low oxygen tensions. The HMM was able to successfully assign gene phenotype classifications to 7316 genes. Our INSeq genetic screen of Rlv3841 grown on glucose and succinate at both 21% and 1% [O_2] enabled the identification of novel transcriptional regulators needed for growth on different carbon sources and oxygen levels.

Growth on succinate also required a significant proportion of cell envelope genes, suggesting restructuring of the cell surface, involving the PrsD-PrsE type I secretion system and EPS production. We carried out an INSeq genetic screen with Rlv3841 inoculated onto pea seedlings and allowed to grow for 5 days in the rhizosphere. Three different mutant libraries were analysed: the input mutants, the mutants retrieved from the rhizosphere, and the root attached mutants. A total of 64-million transposon insertion reads were sequenced, and an insertion density of 88% of all potential mariner insertion sites in the input library was obtained. Subsequent analysis has allowed identification of the genes required for growth specifically in the rhizosphere and genes required specifically for root attachment over 5 days colonisation.

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POSTER 5A-4 /LIGHTNING TALK/

MucR is Required for Transcriptional Activation of Conserved Ion Transporters to Support Nitrogen Fixation of *Sinorhizobium Fredii* in Soybean Nodules

Jian Jiao[1,2,3], Li Juan Wu[1,2,3], Biliang Zhang[1], Yue Hu[1,2,3], Yan Li[1,2,3], Xing Xing Zhang[1,2,3], Hui Juan Guo[1,2,3], Li Xue Liu[1,2,3], Wen Xin Chen[1,2,3], Ziding Zhang[1] and Chang Fu Tian[1,2,3]

[1] *State Key Laboratory of Agrobiotechnology, and College of Biological Sciences, China Agricultural University, Beijing, China*
[2] *Key Laboratory of Soil Microbiology, Ministry of Agriculture, China Agricultural University, Beijing, China*
[3] *Rhizobium Research Center, China Agricultural University, Beijing, China*

To achieve effective symbiosis with legume, rhizobia should fine-tune their background regulation network in addition to activating key genes involved in nodulation (*nod*) and nitrogen fixation (*nif*). Here, we report that an ancestral zinc finger regulator, MucR1, other than its paralog, MucR2, carrying a frameshift mutation, is essential for supporting nitrogen fixation of *Sinorhizobium Fredii* CCBAU45436 within soybean nodules. In contrast to the chromosomal *mucR1*, *mucR2* is located on symbiosis plasmid, indicating its horizontal transfer potential. A MucR2 homolog lacking the frameshift mutation, such as the one from *S. fredii* NGR234, can complement phenotypic defects of the *mucR1* mutant of CCBAU45436. RNA-seq analysis revealed that the MucR1 regulon of CCBAU45436 within nodules exhibits significant difference compared with that of free-living cells. MucR1 is required for active expression of transporters for phosphate, zinc, and elements essential for nitrogenase activity (iron, molybdenum, and sulfur) in nodules but is dispensable for transcription of key genes (*nif/fix*) involved in nitrogen fixation. Further reverse genetics suggests that *S. fredii* uses high-affinity transporters to meet the demand for zinc and phosphate within nodules. These findings, together with the horizontal transfer potential of the *mucR* homolog, imply an intriguing evolutionary role of this ancestral regulator in supporting nitrogen fixation.

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POSTER 5A-5 /LIGHTNING TALK/

Stringent Response-mediated Transcriptional Changes in the *Medicago-Sinorhizobium* Root Nodule Symbiosis

Kathrin Wippel and Sharon R. Long

Department of Biology, Stanford University, Stanford, CA, USA

During the interaction with its host plant *Medicago sativa* (alfalfa), *Sinorhizobium meliloti* encounters several stages of environmental and nutritional changes, which require metabolic and physiological adjustment. Global gene expression in bacteria is altered upon nutrient stress via the stringent response. In general, when sensing uncharged tRNA molecule, the ribosome-bound protein RelA synthesizes the alarmone ppGpp. ppGpp, as well as the transcriptional regulator DksA bind RNA polymerase (RNAP) and shift the lifetime of certain RNAP/promoter complexes, thereby leading to down- and upregulation of specific gene sets.

We have previously shown that both *relA* and *dksA* are important in free-living conditions of *S. meliloti* as well as in symbiosis with alfalfa (1,2). The *relA* mutant phenotypes are very severe: *relA* is indispensible for growth on minimal medium and for nodulation on alfalfa. Detailed transcriptomic analysis on a *relA* mutant strain upon carbon or nitrogen starvation was reported earlier (3). To understand which gene expression changes mediated by the stringent response are necessary and sufficient for the establishment of symbiosis, we performed comparative global transcription profiling via our customized Affymetrix Symbiosis Chip (4) on bacterial wild type, Δ*relA*, and a previously isolated *relA* suppressor strain (5). Cells were grown in culture and induced by the plant flavonoid luteolin. Comparing *relA* to wild type, we found differential regulation of genes for exopolysaccharide biosynthesis, motility, glycine betaine synthesis, and structural proteins. However, no typical symbiosis genes were affected in the mutant; in contrast, *nodE* and *nodG*, as well as the ECF sigma factor gene *rpoE9* were slightly upregulated. There were more transcriptional changes between *relA* and its suppressor than between *relA* and the wild type, suggesting that the suppressor might even overshoot some gene regulation changes. In addition, there are distinct differences between wild type and suppressor, suggesting a lack or addition of specific regulations.

The suppressors initially identified (5) induce nitrogen fixation-deficient nodules on alfalfa, whereas newly isolated suppressors are fix+. All of these suppressors are point mutations in the RNAP β or β' subunits (RpoB or RpoC). We are defining the transcriptomes of three-week old nodules (bacteroids and plant tissue) from the different suppressor types, wild type, and a Δ*relA* strain overexpressing *dksA* (which results in wild type-like behavior), to decipher transcript requirements specific for nodulation and fixation in the context of stringent response. In a second approach, we are assessing the question of which gene transcripts are necessary for symbiosis to occur under ppGpp-deficient conditions by comparing wild type nodules to Δ*relA* mutant-induced nodules on *M. truncatula*, taking advantage of the fact that Δ*relA* nodulates *M. truncatula* in a wild type-like manner.

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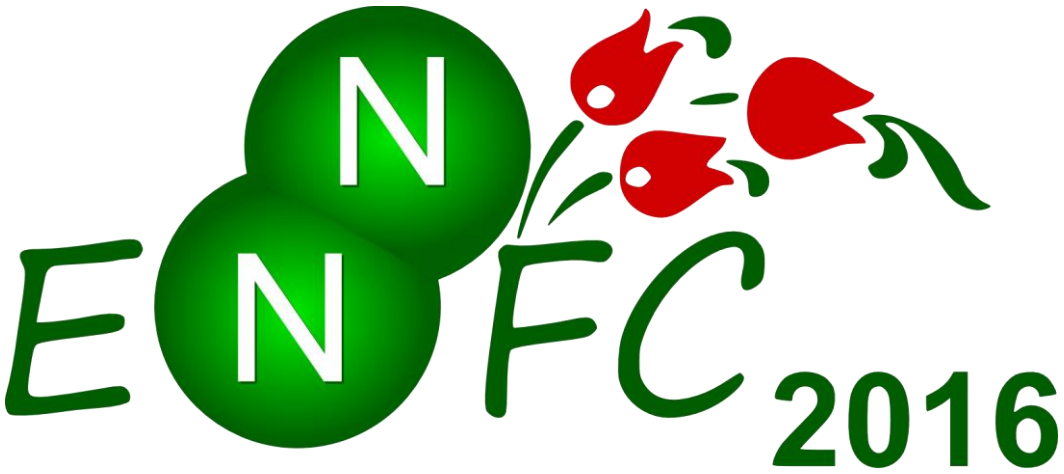

PARALLEL SESSION 6

Biological Nitrogen Fixation in Non-Legume Environments

Chairs: Barbara Reinhold-Hurek, Adriana Hemerly

Endophytic Diazotrophic Bacteria: The Plant Understanding of this Beneficial Association

Thais L.G. Carvalho[1], Helkin G.F. Ballesteros[1], José Paulo M. Filho[1], Pablo Hardoim[1], João Luis Mendes[1], Matheus Atella[1], Thayssa M. D. Fernandes[1], José Ivo Baldani[2], Verônica M. Reis[2], Paulo C. G. Ferreira[1], Adriana S. Hemerly[1]

[1] *Laboratório de Biologia Molecular de Plantas, Instituto de Bioquímica Médica Leopoldo de Meis, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
[2] *Embrapa Agrobiologia, Seropédica, Brazil*

Diazotrophic bacteria have the ability to develop different types of root associations with different plant species. Among the highest rates of BNF are the ones measured in legumes nodulated by endosymbionts. However, it has also been shown that economically important crops, especially monocots, can obtain a substantial part of their N needs from BNF by interacting with associative and endophytic diazotrophic bacteria, that either live near the root surface or endophytically colonize intercellular spaces and vascular tissues of host plants. One of the best-reported outcomes of this association is the promotion of plant growth by direct and indirect mechanisms, as well as increase in tolerance against biotic and abiotic stresses. Inoculants of associative and endophytic diazotrophic bacteria had been shown to lead to positive results on sugarcane yields, which are dependent on the plant genotype and soil conditions.

Our group has been studying sugarcane and maize genes involved in the establishment of a beneficial type of association with nitrogen-fixing bacteria, aiming to assist in the development of more responsive cultivars to inoculants of beneficial diazotrophs. An integrated differential transcriptome was generated by Illumina RNAseq and it provided an overview of sugarcane and maize metabolism, growth and development controlled by nitrogen, water and endophytic nitrogen-fixing bacteria during a successful association. All together, the data suggest that an important control of the efficiency of the association is already set in the early stages of plant-bacterium recognition, when specific plant genotypes sense the environment and regulate several plant signaling pathways involved in microorganism recognition and plant defense.

We propose that "soil-rhizosphere-rhizoplane-endophytes-plant" could be considered as a single coordinated unit with dynamic components that integrate the plant with the environment to generate adaptive responses in plants to improve growth. The homeostasis of the whole system should recruit different levels of regulation, and recognition between the parties in a given environment might be one of the crucial factors coordinating these adaptive plant responses.

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A Glance at the Endophytic Lifestyle of *Azoarcus* sp. BH72: Factors Contributing to Endophytic Competence

Andreas Beust, Theresa Dinse, Abhijit Sarkar and Barbara Reinhold-Hurek

Institute of Microbe-Plant Interactions, Faculty of Biology and Chemistry, University of Bremen, Germany

The diazotrophic model endophyte of grasses, *Azoarcus* sp. strain BH72, colonizes roots of its original host plant Kallar grass and rice in similar patterns. The lifestyle of these endophytes is remarkable, as they establish in the apoplast of plants in high numbers; however the molecular mechanisms by which they interact with their host are not yet well understood. How do bacteria adapt to their endophytic lifestyle in comparison to free-living growth, and which proteins do they require for endophytic competence (1)? Availability of the genome of strain BH72 (2) allows application of functional genomic analyses during interaction.

The type VI secretion system (T6SS) is the most recently described secretion system found in ~25% of all Gram-negative bacteria. Its involvement in the interaction between different bacteria or between bacteria and their eukaryotic hosts has been studied intensively over the last years. However, non-pathogenic interactions have scarcely been analyzed. The genome of *Azoarcus* contains two gene clusters encoding for putative T6SSs, termed *sci* and *imp*. Secretion of the T6SS hallmark protein HCP was shown, and initial experiments pointed towards a negative influence on plant colonization (3). Here we unraveled the T6SS functions in more detail.

Proteomic studies in wild type and mutant cells showed that mainly the Sci-system was active in protein secretion (as detected by Hcp proteins in the supernatant). To carry out mass spectrometric analyses of the T6SS-dependent secretome, a mutant carrying gene disruptions in both, *sci* and *imp* system, was compared to a hyper-secreting mutant of strain BH72. It was generated by inactivation of *tagF,* a homolog to a *Pseudomonas aeruginosa* gene encoding an inhibitor of T6SS protein secretion. Several putatively secreted proteins could be identified. However, the Imp-system did not secrete its cognate Hcp protein, although the respective genes were strongly expressed under conditions of nitrogen fixation. Even the hyper-secreting mutant showed no secretion of Imp-related proteins. Surprisingly, *Azoarcus* sp. carrying a gene knockout in this apparently inactive Imp-system showed a strong reduction in endophytic rice root colonization. We will discuss novel cues which might activate secretion by this T6SS system. Additional bacterial factors that contribute to endopyhtic colonization will also be discussed.

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Discovery of a Novel Rhizopine Synthesis Pathway Paves the Way for Synthetic Symbioses and Nitrogen Fixing Cereal Crops

Barney A Geddes[1], Amelie Joffrin[2], Ponraj Paramasivan[3], Giles Oldroyd[3], Stuart J. Conway[2] and Philip S. Poole[1]

[1] *Department of Plant Sciences, University of Oxford, Oxford, United Kingdom*
[2] *Department of Chemistry, University of Oxford, Oxford, United Kingdom*
[3] *Department of Cell and Developmental Biology, John Innes Centre, Norwich, United Kingdom*

E-mail: barney.geddes@plants.ox.ac.uk

The engineering of cereal crops that can fix atmospheric nitrogen, like in the rhizobium-legume symbiosis, has been a dream for decades. Recently there has been a renewed interest in this area of research and several approaches have arisen with the common aim of transferring nitrogen fixation to cereal crops (1, 2). The Synthetic Symbiosis project aims to use bacteria with the pre-existing ability to efficiently colonize cereals as chassis to engineer nitrogen fixation (3). One of the critical aspects of a functional symbiosis is the ability to establish communication between the two partners. As part of this project we aim to exploit specialized aminocyclitol compounds called rhizopines to serve as part of a signal exchange between an engineered plants and microbes. Rhizopines also have important implications for selectively mediating the growth of engineered microbes by creating biased rhizospheres in cereals that have been engineered to produce them.

Rhizopines are normally produced by a small subset of rhizobial species during symbiosis with legume crops (4). Using biochemical assays and comparative analytical chemistry we have elucidated a novel pathway for rhizopine synthesis. These findings have contributed significantly to the understanding of rhizopine synthesis in its natural context, as well as allowed the genetic transfer of rhizopine biosynthesis to cereal crops. Further, we have developed bacterial biosensors that can respond to rhizopine and specifically activate gene expression in bacteria. Together these technologies have allowed us to achieve the first steps required to coordinate communication within a synthetic symbiosis between bacteria and plants.

This work is supported by UK-BBSRC and US-NSF joint funding.

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POSTER 6-1 /LIGHTNING TALK/

The RNA Chaperone Hfq is a Global Regulator in the Nitrogen-Fixing *Pseudomonas stutzeri* A1501

Wei Wang[1], Yuhua Zhan[1], Wei Lu[1], Claudine Elmerich[2], Yongliang Yan[1], Min Lin[1]

[1] *Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China*
[2] *Institut Pasteur, Paris, France*

The nitrogen-fixing *P. stutzeri* A1501, isolated from the rice rhizosphere in China, fixes nitrogen due to the acquisition of a 49-kb nitrogen fixing island (1). Transcriptome analysis under nitrogen and oxygen limitation, or after ammonia shock, revealed dramatic changes in expression patterns of a number of genes related to carbon and nitrogen metabolism, located both in the *nif*-island and in the core genome, suggesting complex regulatory networks (2, 3).

The RNA chaperone Hfq was reported to be involved in many intracellular metabolic processes by promoting the interaction between protein or non-coding RNA and target mRNA, regulating the stability of mRNAs (4). An *hfq* gene was identified in the A1501 genome. Disruption of *hfq* did not affect the growth of *P. stutzeri* A1501 in rich or minimal medium. The *hfq* mutant strain was found to be impaired in several phenotypes including decrease resistance to oxidative and osmotic stresses, increased biofilm formation ability and decreased denitrification ability when nitrite was used as the electron acceptor. Real-time RT-PCR revealed significant decreased expression of the catalase *katA/B/E/G*, alkyl hydroperoxide reductase *ahpF/C* and glutathione peroxidase encoding genes, as well as decreased expression of the *nirB*, *nirS* encoding the key cytochrome C-552, cytochrome *cd1* nitrite reductase precursor and *nosR* encoding NosR regulatory protein.

We also found that *hfq* was involved in the regulation of nitrogen fixation, as its inactivation caused a significant decrease in nitrogenase activity. qRT-PCR revealed that expression of *nifA*, *nifK*, *glnA*, *glnK, rpoN* and *ntrC* were significant downregulated in the *hfq* mutant. Furthermore, Western blot analysis revealed a decreased amount of NifD and NifK polypeptides.

The above results showed that Hfq is involved in various physiological processes in *P. stutzeri* A1501. Further research will focus on the molecular mechanism of Hfq in regulation the expression of specific target genes.

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POSTER 6-2 /LIGHTNING TALK/

Identification and Functional Characterization of Genes Involved in Carbon Source Utilization in *A. brasilense* Sp7

Vijay Shankar Singh, Ashutosh Prakash Dubey, Ankush Gupta, Sudhir Singh and Anil Kumar Tripathi.

School of Biotechnology, Banaras Hindu University, Varanasi, India

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Azospirillum brasilense is nitrogen fixing, non-photosynthetic, plant growth promoting ά-proteobacteria found in the close vicinity of various plant roots (1) including C3 cereals and C4 grasses. Organic acids (mainly C4-dicarboxylates) as well as trace amount of sugars and sugar alcohols of root exudates act as preferred source of carbon and energy for the nitrogen fixation and for the growth (2). To search the genes and proteins involved in C4-dicarboxylates utilization, 2D gels of *A. brasilense* grown in different C4-dicarboxylates supplemented medium have been resolved and observed that a DctP protein is upregulated in malate grown culture while nearly constant expression has been observed in succinate and fumarate grown cultures of *A. brasilense*. Insertional inactivation of induced *dctP* and double knock-out mutant of *dctP* and another C4-dicarboxylates transporter *dctA* have shown that DctP is a major transporter (~75% growth retardation in *dctP::km* mutant) while DctA (~25% growth retardation in *dctA::gm* mutant) is a minor C4-dicarboxylates transporter in *A. brasilense*. Enhanced promoter activity of *dctP* and *dctA* at µM and mM range of substrates, respectively have been shown that DctP is high affinity while DctA is low affinity transporter and enhanced promoter activity of *dctP* in *σ54::km* while zero activity of *dctA* promoter have been shown that σ54 positively regulate the expression of *dctA* gene. In addition to C4-dicarboxylate transporters we observed a PQQ dependent quinoprotein alcohol dehydrogenase (ExaA) protein has been upregulated in glycerol as well as fructose grown cultures of *A. brasilense* and belongs to Type-I of PQQ-ADH. 5' RACE study predicted the σ54 binding site and it has been demonstrated that σ54 regulate positively and another RpoH2 sigma factor regulate negatively the expression of *exaA* gene. Role of divergently organized two component system as well as regulator binding site have been predicted by mutations and it has been shown that LuxR type of regulator EraR regulate the expression of *exaA* by showing positive interaction with promoter bounded σ54-RNA Polymerase complex without having GAFTGA domain.

References:

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POSTER 6-3 /LIGHTNING TALK/

Development of Tools for Transformation and Gene Expression in *Paenibacillus* Species and Complete Genome Sequence of *Paenibacillus riograndensis* SBR5

Luciana Fernandes de Brito[1], Marta Irla[1], Tatjana Walter[1] and Volker F. Wendisch[1]

Chair of Genetics of Prokaryotes, Faculty of Biology & CeBiTec, Bielefeld University, Bielefeld, Germany

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Paenibacillus riograndensis is a rod-shaped, Gram-positive, nitrogen-fixing bacterium. The strain SBR5 was isolated from the rhizosphere of wheat plants cultivated in Rio Grande do Sul, Brazil. In addition to nitrogen fixing, SBR5 possesses further plant growth promoting activities, e.g. production of indol-3-acetic acid and siderophores. Thus, *P. riograndensis* SBR5 is interesting for agricultural purposes, although it has not been studied much.

Here, we have determined the complete genome sequence of SBR5 and completely annotated the genome. The genome consists of one chromosome with 7.893.056 bps, containing 6705 protein coding genes, 87 tRNAs and 27 rRNAs (1).

We also developed a new transformation protocol for *Paenibacillus* species based on physical permeation through mixing the cell suspension with a plasmid-aminoclay solution (2). Transformation was shown by plasmid isolation and re-transformation as well as by heterologous production of a fluorescent reporter protein. Furthermore, the *gfpUV* reporter gene was used to test rolling-circle and theta-replicating plasmids for constitutive and inducible expression. Fluorescence activated cell scanning (FACS) verified the versatility of the developed expression vectors for constitutive and graded inducible expression. These gene expression systems could be transferred to another Paenibacillus species, i.e. *P. polymyxa*. In addition, inducible gene expression was applied to metabolic engineering of *P. riograndensis*.

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POSTER 6-4 /LIGHTNING TALK/

The Regulation of Nitrogen Fixation and Assimilation in the Associative Diazotroph *Klebsiella oxytoca* M5a1

Christopher J. Waite, V. Elsin, R. Berchtold, T. Tanny, J. Gan, N. Sanguankiattichai, S. Amartay, M. Buck, J. Schumacher.

Division of Cell and Molecular Biology, Department of Life Sciences, Imperial College Road, Imperial College, London, United Kingdom

Engineering free-living and associative diazotrophic bacteria for the release of fixed nitrogen (N) into the rhizosphere represents one promising strategy for meeting the global demand for agricultural fertiliser sustainably (1-2). As part of an ongoing collaborative project (BB/N003608/1) aiming to rationally re-engineer the cell signalling and metabolism of model diazotrophs for the supply of surplus reduced N (e.g. ammonium) to plants, we present a preliminary model for the regulatory interplay between N fixation and N assimilation in the associative, soil-dwelling bacterium *Klebsiella oxytoca* M5a1 (*Ko*). In most bacteria the rate of N assimilation is coupled to the cellular N status (glutamine/α-ketoglutarate ratio) by a regulatory cascade involving post-translational uridylylation of PII type proteins (GlnB and GlnK) and the σ^{54}-type transcriptional activator NtrC (3). In diazatrophs such as *Ko*, these proteins also regulate the expression of the *nif* gene cluster, encoding the nitrogenase complex and its associated factors, via a second, downstream σ^{54}-type transcriptional activator NifA (4). Nitrogenase expression is coupled tightly to internal N status (via NtrC), anaerobiosis (via NifL, the negative regulator of NifA) and import of exogenous fixed N (via interactions between GlnK and the primary ammonium transporter, AmtB (5)). Ultimately, the co-regulation of the *gln* (N assimilation) and *nif* (N fixation) regulons by multifunctional regulatory proteins affords highly economical nitrogen metabolism, according to supply and demand, in which a surplus of fixed N compounds is minimised.

Ko provides a suitably characterised model diazotroph in which to develop an integrated systems-level understanding of N economy management, including identification of key nodes of control and robustness, whether catalytic or regulatory, that prevent excess fixed N production and export. In preparation for omics analyses (RNA-seq, targeted MRM-MS proteomics and LC-MS metabolite profiling) we have characterised multiple key parameters during the diazatrophy transition that follows ammonium run-out including (a) cell growth rate, (b) transcription of key N regulons, (c) nitrogenase activity and (d) critical O_2 concentrations. We have developed an O_2-independent fluorescent gene reporter system and a library of regulatory mutants (both gene deletions and CRISPR-targeted substitutions) which together reveal novel control mechanisms at play in this organism. We report initial findings employing synthetic transcription factors, for instance a chimera of the O_2-sensitive *Bradyrhizobium japonicum* and native *Ko* NifA homolgoues, to redirect/tune *nif* gene expression and thereby uncouple the N demand of the cell from N fixation activity.

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POSTER 6-5 /LIGHTNING TALK/

Engineering a Biased Plant Rhizosphere to Establish Synthetic Symbioses in Cereals

Ponraj Paramasivan[1], Barney A. Geddes[2], Philip Poole[2] and Giles Oldroyd[1]

[1] *Department of Cell and Developmental Biology, John Innes Centre, Norwich, United Kingdom*
[2] *Department of Plant Sciences, University of Oxford, Oxford, United Kingdom*
E-mail: ponraj.paramasivan@jic.ac.uk

One strategy to engineer plant-microbe interactions in the rhizosphere to enhance crop production in future agroecosytems is to create biased rhizospheres that benefit the plant. A well-known natural instance of a biased rhizosphere is the opine concept during pathogenic *Agrobacterium* - plant interactions. In this example plant production of opines benefit the *Agrobacterium* strains that carry the genes for opine catabolism. Since engineering opine synthesis into the rhizosphere will enrich phytopathogenic agrobacteria, an alternative plant-produced carbon substrate to engineer a biased rhizosphere is rhizopine. Rhizopines are nodule specific, opine-like compounds synthesized by a few rhizobial strains in root nodules and exuded into the rhizosphere. Rhizopine give plant-nodulating rhizobia a nutritional advantage in the rhizosphere over other saprophytic microorganisms. Current advances in plant and microbial synthetic biology will enable us to use metabolic engineering to create a rhizopine-based biased rhizosphere and establish synthetic symbioses for enhanced nitrogen provision to cereals.

We aim to engineer LCO inducible production of rhizopines as a novel substrate in cereal roots (barley) that can specifically support nitrogen fixation by genetically modified, rhizopine catabolising bacteria which carry the ability to fix some level of nitrogen in the free-living state. In this context we are analysing the constitutive and inducible (Nod factor) production of rhizopines in transient plant transformation systems so that this approach can be transferred to cereals.

This work is supported by UK-BBSRC and US-NSF joint funding.

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[1] *Rogers C. and Oldroyd G. (2014) J. Exp. Bot. 65(8): 1939-1946.*
[2] *Geddes et. al. (2014) Curr. Opin. Biotechnol. 32: 216-222.*

POSTER 6-6 /LIGHTNING TALK/

Signaling Pathway in the Actinorhizal Root Nodule Symbiosis

Sergio Svistoonoff[1,6], Valérie Hocher[1], Leandro Imanishi[2], Laurent Brottier[1], Elodie Pirolles[1], Daniel Moukouanga[3], Mireille Chabaud[4], Joëlle Fournier[4], David Barker[4], Luis Wall[2], Louis Tisa[6], Didier Bogusz[3], Claudine Franche[3], Hassen Gherbi[1]

[1] *IRD-LSTM, Campus de Baillarguet, Montpellier, France*
[2] *Laboratorio de Bioquímica, Microbiología e Interacciones Biológicas en el Suelo, Universidad Nacional de Quilmes R. Sáenz Peña 352, Bernal, Argentina*
[3] *IRD, UMR DIADE, Campus Agropolis, Montpellier, France*
[4] *LIPM, INRA (UMR 441), CNRS (UMR 2594), Castanet-Tolosan, France*
[5] *Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire, Durham, United States*
[6] *Laboratoire Commun de Microbiologie IRD/ISRA/UCAD, Sénégal*

Legumes and a phylogenetically diverse group of species called actinorhizal plants are able to establish N_2-fixing Root Nodule Symbiosis (RNS) in association with rhizobia and *Frankia* bacteria, respectively. Legumes and actinorhizal plants all belong to the Fabid Clade suggesting that the ancestor of extant Fabids acquired a genetic predisposition towards nodulation. However the genetic basis of this predisposition is still unknown. In model legumes, perception of rhizobial signals (Nod factors) by LysM-RLKs receptors activates a signaling cascade that then leads to the formation of root nodules. Part of this pathway is also essential for the arbuscular mycorrhizal (AM) symbiosis and is referred to as the Common Symbiotic Signaling Pathway (CSSP).

We are investigating the genetic bases of symbiotic signaling in actinorhizal symbioses. Using transcriptomic and functional studies, we discovered that many genes involved in the CSSP required for nodulation and mycorrhiza formation in legumes are also present in actinorhizal species and showed that at least two of these, *SymRK* and *CCaMK*, are essential for the establishment of actinorhizal symbiosis (1). In addition, we demonstrated that *NIN* gene, a transcription factor required for rhizobium-legume symbiosis is also essential for nodulation in the actinorhizal species *Casuarina glauca,* thus unveiling the first element of a common nodulation-specific pathway (2). Furthermore, by developing sensitive bioassays, we revealed that cell-free culture supernatants of *Frankia* CcI3 strain are able to induce both sustained high frequency Ca^{2+} spiking and transcriptional activation of Pro*CgNIN:GFP* in root hairs of *C. glauca*. Based on these two bioassays, we showed that biological active fraction present in *Frankia* CcI3 supernatant is hydrophilic, of low molecular weight and resistant to chitinase degradation (3).

Altogether, these studies provide new insights both into the molecular dialogue between *Frankia* and actinorhizal species, and into the evolution of RNS.

References:

[1] *Svistoonoff et al. (2014) Curr. Opin. Plant Biol. 20: 11-18.*
[2] *Clavijo et al. (2015) New Phytol. 208(3):887-903.*
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POSTER 6-7 /LIGHTNING TALK/

Functional Genomics of Cyanobacteria in Symbiosis with Boreal Feather Mosses

Denis Warshan[1], Christopher L. Dupont[2], Philip D. Weyman[3], Ulla Rasmussen[1]

[1] *Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, Sweden*
[2] *Microbial and Environmental Genomics Group, J. Craig Venter Institute, La Jolla, United States*
[3] *Synthetic Biology and Bioenergy Group, J. Craig Venter Institute, La Jolla, United States*

The nitrogen cycling in boreal forest ecosystems is largely determined by a symbiotic association between feather mosses (*Pleurozium schreberi* and *Hylocomium splendens*) and diazotrophic cyanobacteria that fix majority of nitrogen flowing into boreal ecosystems (1). Because nitrogen is often limiting in boreal forests, the interaction between the cyanobacteria and the mosses greatly affects the productivity of this ecosystem that makes up almost 30% of Earth's forested land (2-3). We seek to understand the genetic diversity of the cyanobacteria associated with the mosses and the molecular steps leading to the moss-cyanobacterial symbiosis.

We sequenced the genomes of five different *Nostoc* spp. that are able to form symbiotic associations with feather moss. As a control, we also sequenced the genome of one *Nostoc* sp. that is unable to form symbioses with the mosses. Comparative genome analysis of these cyanobacterial species allowed us to probe the genomic diversity of moss-associated *Nostoc* strains and identify a set of 32 genes differentially retained in genomes of symbiotic competent cyanobacteria compared to the non-symbiotic competent strain.

We also obtained transcriptomic and proteomic data for *Nostoc* grown in isolation, together, or with chemical contact with the moss only through filter separation. Transcriptomic and proteomic data revealed that differentially retained genes and their neighborhood are upregulated in chemical contact (gas vesicles, chemotaxis related genes), during both condition (pyrroloquilonine-quinone and exopolysaccharide production) and together with the moss partner (taurine catabolism and aliphatic sulfonate transporter). Thus, we hypothesize that cyanobacteria symbiotic gene clusters are essential to establish the symbiosis with feather moss.

References:

[1] *DeLuca et. al. (2002) Nature 419:917–920.*
[2] *Anderson et al. (1991) Ecol. Appl. 1:326–347.*
[3] *Goodale et al. (2002) Ecol. Appl. 12:891–899.*

POSTER 6-8 /LIGHTNING TALK/

Evolution of the Actinorhizal Symbiosis: Analysis of Bacterial Genomes of the Basal Cluster

Than Van Nguyen and K. Pawlowski

Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, Sweden

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The ability to establish root nodule symbioses is restricted to four different plant orders, the Fabales, Fagales, Cucurbitales and Rosales which together form the fabid clade. With the exception of *Parasponia* sp., all host plants of rhizobia symbiosis belong to the order Fabales. The other three orders contain a diverse group of plants within eight different families that enter into nitrogen-fixing symbiosis with soil actinobacteria of the genus *Frankia.* Phylogenetically, symbiotic *Frankia* strains can be divided into three main clusters. Members of cluster II nodulate the broadest range of host plants with species from four families from two different orders, growing on six continents. This cluster also forms the basal group of the genus *Frankia.*

Of the three genomes available from cluster II, two originated in Asia - Dg1 from Pakistan (1), BMG5.1 from Japan (2) – and one from North America – Dg2 from California, USA (unpublished). Based on average nucleotide identity analysis, BMG5.1 and Dg1 represent the same species, while Dg2 represents another species. This indicates that there is less species diversity in cluster II than in clusters I or III. Cluster II strains contain features not present in the other two clusters, e.g., Dg1 and Dg2 (but not BMG5.1) contain the canonical *nod* genes *nodABC* which are expressed in nodules. All cluster II strains contain several copies of the mammalian cell entry (*mce*) genes which encode steroid transporters in other actinobacteria. These and other features of cluster II genomes will be discussed.

References:

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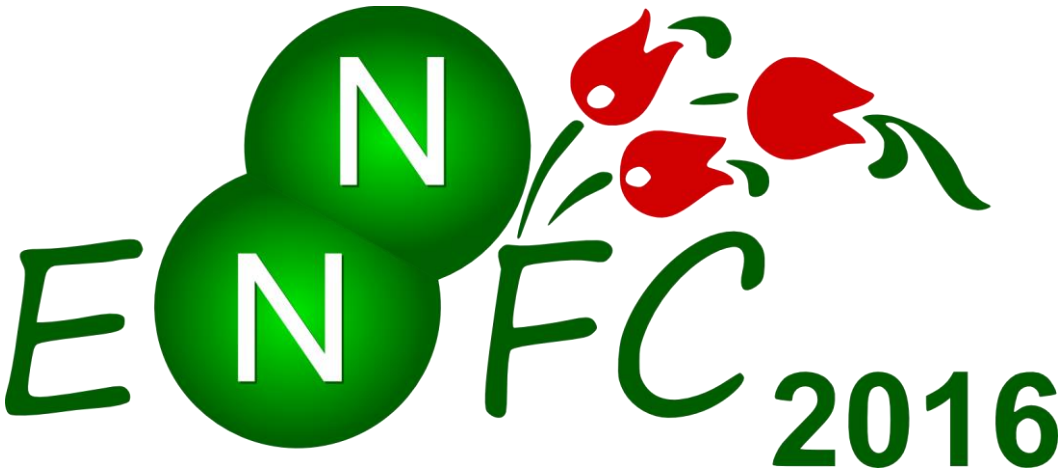
[2] *Gtari et al. (2015). Scientific Reports 5: 13112.*



PARALLEL SESSION 5B

Functioning of the N-fixing Symbioses /Plants/

Chairs: Katharina Pawlowski, Jean-Michel Ané

Epigenetic Regulation is Essential for the Development of Indeterminate Nodules

Carine Satgé[1], Sandra Moreau[1], Erika Sallet[1], Marie-Christine Auriac[1], Gaëlle Lefort[2], Céline Remblière[1], Céline Noirot[2], Marie-Françoise Jardinaud[1], Pascal Gamas[1]

[1] *Laboratoire des Interactions Plantes-Microorganismes (LIPM), Université de Toulouse, INRA, CNRS, Castanet-Tolosan, France*
[2] *Plateforme Bioinfo Genotoul- Unité MIAT, INRA, Castanet-Tolosan, France*

The formation of root nodules requires coordinated plant and bacterial cell differentiation, a developmental transition which requires major changes in plant gene expression, the control of which is still poorly understood. In indeterminate nodules formed in *Medicago truncatula* and other temperate legumes, a large family of genes encoding nodule-specific cysteine-rich (NCR) peptides is strongly upregulated during nodule development and has been shown to play a key role in bacteroid differentiation (1-4).

We recently described an approach based upon RNAseq and laser microdissection of *M.truncatula* nodule zones which has allowed us to correlate gene expression with distinct nodule developmental stages (5). Using these data, we have discovered that several genes involved in the control of DNA methylation are spatially regulated within the nodule. For example, DNA methylation genes are well expressed in the apical nodule zones but downregulated in the differentiation and the nitrogen fixation zones. By contrast, the DEMETER (DME) DNA demethylase, known for its essential role during seed development in several plant species, is specifically expressed in the differentiation zone of mature nodules and strongly upregulated in nodules as compared to roots. The orthologous *DME* gene was also found to be activated in pea and alfalfa nodules but not in *Lotus japonicus* and soybean determinate nodules. Time-course and symbiotic mutant analyses indicate that Mt*DME* expression is highly correlated with nodule differentiation, while RNA interference approaches demonstrate that Mt*DME* is critical for the differentiation of plant cells and bacteroids and consequently for the formation of nitrogen-fixing nodules. Bisulphite sequencing coupled to the capture of approx. 10 Mb of selected genomic regions have allowed us to identify thousands of cytosine residues exhibiting demethylation in nodules, located notably in or adjacent to *NCR* genes. This underlines the importance of epigenetic regulation throughout indeterminate nodule development.

References:

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[4] *Horváth et al. (2015) Proc Natl Acad Sci U S A, 112, 15232-15237.*
[5] *Rouxet al. (2014) Plant J 77, 817-837.*

Dynamic Changes in Chromatin Structure During Endoreduplication Regulate Expression of Nodule-specific *NCR* Genes in *Medicago truncatula*

Marianna Nagymihály[1,2], Federico Ariel[3], Teddy Jégu[3], Attila Szűcs[1], Moussa Benhamed[3], Attila Kereszt[1], Peter Mergaert[2] and Éva Kondorosi[1,2]

[1] *Institute of Biochemistry, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary*
[2] *Institut de Biologie Intégrative de la Cellule, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France*
[3] *Institute of Plant Sciences Paris-Saclay (IPS2), CNRS, INRA, Univ Paris Sud, University of Evry, University of Paris-Diderot, Sorbonne Paris-Cité, University of Paris-Saclay, Gif-sur-Yvette, France*

Medicago truncatula Nodule-specific Cysteine Rich (NCR) peptides are plant effectors that direct the differentiation of *Rhizobium* bacteria into nitrogen-fixing bacteroids within the symbiotic nodule cells. The formation of these cells is driven by endoreduplication and is associated with transcriptional reprogramming, including the induction in two waves of hundreds of *NCR* genes which are transcriptionally silent in all other plant tissues. Here, we show for three selected *NCR* genes that transitions in ploidy level during the differentiation of the symbiotic nodule cells correlate with *NCR* gene activation or repression, with changes in specific histone modifications of the genes and with opening or closing of their chromatin structure but not with an alteration of the DNA methylation level. Our results show that histone H3 lysine 27 trimethylation (H3K27me3) is one of the major determinants of the nodule-specific *NCR* gene expression. The coexistence of the silencing H3K27me3 and the activating histone H3 lysine 9 acetylation (H3K9ac) chromatin marks maintain a repressed state of the *NCR* genes but poise their expression, allowing their activation by removing the H3K27me3 marks in the presence of symbiotic nodule cell differentiation signals.

Medicago truncatula Nodule-Root (*noot*) Genes Are Guards of the Symbiotic Organ Identity

Kevin Magne[1], Jean Malo Couzigou[2], Jeoffrey Georges[1], Igor Tikhonovich[3], Vladimir Zhukov[3], Pascal Ratet[1]

[1] *IPS2 : Institut des Sciences des Plantes de Paris Saclay, UMR Université Paris Sud - INRA 1403 - CNRS 9213 - Université Paris Diderot - Université Evry Val d'Essonne, Bat 630 - Orsay, France*
[2] *ETH Zurich, Agronomy, Plant Protection and Animal Health, Agricultural Plant Science, Zürich, Switzerland*
[3] *All-Russia Research Institute for Agricultural Microbiology, Laboratory of Genetics of Plant-Microbe Interactions, St. Petersburg, Russia*

Symbiotic interaction between legume plants and soil bacteria results in the formation of the nitrogen fixing nodule. It is supposed that root and nodule developments share common features. We have identified the *Medicago truncatula NODULE ROOT* (*NOOT*) and *Pisum sativum COCHLEATA* (*COCH*) orthologous genes as necessary for the robust maintenance of nodule identity (Couzigou et al., 2012). In the *noot* and *coch* mutant plants, roots develop from the extremities of the nodule vascular tissues. A second *NOOT* gene (*NOOT2*) is present in *M. truncatula*. The expression of this gene is induced during symbiosis but the *noot2* mutant shows no symbiotic phenotype. In contrast, the corresponding *noot1noot2* double mutant shows enhanced nodule to root conversion. In this double mutant, the reduction in symbiotic organ identity results in the formation of non-functional highly modified symbiotic organs with fix$^-$ phenotype. The characterization of these genes thus highlights the root evolutionary origin of vascular strands in the symbiotic nodule, and suggests that the *NOOT* and *COCH* genes were recruited to repress root identity and coordinate the development of the legume symbiotic organ. In order to investigate a potential relation between *NOOT* and the presence of a persistent meristem, we identified the *NOOT* ortholog in *Lotus japonicus* (a determinate nodule forming legume). We are now characterizing the corresponding insertion mutant.

The role for these two *NOOT* genes as well as their orthologs in legume development and more precisely in the nodule symbiotic organ identity will be discussed.

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Legume Shoots Induce a 24 H Nitrogenase-activity Rhythm under the Influence of Various Environmental Cues by a Common Molecular Mechanism

Joachim Schulze[1], Ricardo A. Cabeza[2], Rebecca Liese[3]

[1] *Department of Crop Science, Section for Plant Nutrition and Crop Physiology, Faculty of Agriculture, University of Goettingen, Goettingen, Germany*
[2] *Universidad de Chile, Facultad de Ciencias Agronómicas, Departamento de Ingeniería y Suelos, Santiago de Chile, Chile*
[3] *Department of Plant Ecology and Ecosystems Research, Faculty of Biology, Goettingen, Germany*

Symbiotic nitrogen fixation is known to be down-regulated when environmental cues restrict growth or when alternative N-sources (soil-N or N remobilization from old leaves) become available. Through a system that allows the continues and non-invasive measurement of symbiotic nitrogenase activity (1), we found that the down-regulation of nitrogenase is initially achieved by the induction of a 24 h rhythmic nitrogenase-activity pattern while under undisturbed conditions the activity during the 24 h period is constant. The pattern of the rhythm is conserved over treatment as diverse as nitrate- or ammonium application, P- or Mg-deficiency, continues darkness and, interestingly, by hypernodulation. We will show that the rhythm is clearly shoot induced. The most convincing argument for that is the fact that in a split-root system nitrate or ammonium induces the rhythm in both, a treated and the corresponding untreated root/nodule part with a striking similarity in the structure of the pattern. Indirect evidence suggests that the shoot impact on the nodules is initiated when the leave N-needs are satisfied. Comparative nodule transcriptome-analysis (RNAseq) revealed that the molecular mechanisms that induce the rhythm in the nodules are common among the diverse treatments. For example, during a strong decline in activity in the afternoon, genes for nodule specific cysteine-rich peptides (NCR), leghemoglobins and other late nodulins are concerted and strongly down-regulated and the inner nodule iron allocation appears to be disturbed.

References:

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[2] *Cabeza et al. (2014) Plant Physiol. 164:400–411.*

POSTER 5B-1 /LIGHTNING TALK/

The Role of U-box Ubiquitin Ligases during Plant-Microbe Interactions

Ernö Kiss, Boglárka Oláh, Zoltán Bozsóki, Andrea Borbola, Erzsébet Fehér-Juhász, Sándor Jenei and Gabriella Endre

Biological Research Centre of the Hungarian Academy of Sciences, Institute of Genetics, Szeged, Hungary

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Post-translational modifications represent very rapid and efficient systems in the precise regulation of the function of different proteins. Ubiquitination of proteins is a modification that can significantly change the fate of a protein. It can target the protein for degradation to the proteasome or can change its activity or location within the cell either. The E3 ubiquitin ligases - one of the three enzymes needed for linking the ubiquitin to the protein targets - are of particular interest in this process as they confer substrate specificity, but also, they can identify several target proteins for ubiquitination. In plants, the U-box domain containing E3 family has undergone a large gene expansion that may be attributable to biological processes unique to the plant life cycle. Research on these genes from several different plants has started to elucidate a range of functions for this family, from self-incompatibility and hormone responses to defence and abiotic stress responses, and they also could be identified as being essential for nitrogen-fixing symbiosis.

We work on the characterization of two different kinds of U-box containing E3 ligases that have roles in the process of nitrogen-fixing symbiosis in *Medicago truncatula*. Beside the functional U-box driving the link from the E2 enzyme onto the target protein, they contain ARM-repeats, but the rest of the modules are different. Their E3 ligase activity has been confirmed in biochemical experiments, on the other hand their in vivo detection and examination is very difficult due to their unstable nature. In symbiotic plant tests we have characterized their mutant phenotype as well as the effect of their over-expressions. Their homologs from other plants were also recognized and cloned. When truncated form of these E3 ligases or their homologs were introduced into the mutant or wild type plants, the resulting phenotype allowed to distinguish the possible function of the different domains.

POSTER 5B-2 /LIGHTNING TALK/

MtNramp1, MtZIP6, and MtCOPT1 are Respectively Responsible for Iron, Zinc, and Copper Uptake by *Medicago truncatula* Nodule Cells

Manuel Tejada-Jiménez[1], Ángela Saéz[1], Marta Senovilla[1], Rosario Castro-Rodríguez[1], Isidro Abreu[1], Viviana P. Escudero[1], Igor Kryvoruchko[2], Mercedes M. Lucas[3], Michael Udvardi[2], Juan Imperial[4], Manuel González-Guerrero[1]

[1] *Centro de Biotecnología y Genómica de Plantas, Campus de Montegancedo, Universidad Politécnica de Madrid, Madrid, Spain*
[2] *Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, United States*
[3] *Instituto de Ciencias Agrarias, Consejo Superior de Investigaciones Científicas, Madrid, Spain*
[4] *Consejo Superior de Investigaciones Científicas, Madrid, Spain*

Legume nodules require relatively large amounts of transition metals (iron, copper, zinc…) as cofactors of some of the key enzymes involved in symbiotic nitrogen fixation (1). These metals are provided by the host plant through the vasculature and, in the case of indeterminate type nodules, released in the infection/differentiation zone of the nodule (2). Consequently, a number of metal transporters must be in place to introduce these elements into the nodule cells to be later delivered to the symbiosomes.

We have identified three *Medicago truncatula* metal transporters encoding genes, *MtNramp1* (3), *MtZIP6*, and *MtCOPT1*, that are expressed at high levels in the nodule zone where metals have to be incorporated from the apoplast. Yeast complementation assays show that they are respectively involved in iron, zinc, and copper transport towards the cytosol. Consistent with a role in metal uptake from the apoplast, they are located in the plasma membrane of nodule cells. *Knock out* or *knock-down* plants of each gene have reduced growth and reduced nitrogenase activity. These phenotypes were restored by reintroducing a wild type form of the mutated gene or by watering the plants with a nutritive solution fortified in the corresponding transported metal. These results are consistent with a model in which MtNramp1 is responsible for Fe^{2+} loading of nodule cells, while MtZIP6 is for Zn^{2+} uptake, and MtCOPT1 for Cu^{+}.

This work was supported by ERC Starting Grant (ERC-2013-StG-335284) and MINECO Grant (AGL-2012-32974) to MGG.

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[3] *Tejada-Jiménez et al. (2015) Plant Physiol. 168: 258-272.*

POSTER 5B-3 /LIGHTNING TALK/

Thioredoxin 1 s1 is Essential for Bacterial Terminal Differentiation in the Nitrogen-fixing Symbiosis in *Medicago truncatula*

C. Ribeiro Werner[1], F. Baldacci-Cresp[1], O. Pierre[1], M. Larousse[1], G. Alloing[1], P. Mergaert[2], P. Gamas[3], N. Rouhier[4], F. Montrichard[5], P. Frendo[1] and Eric Boncompagni[1]

[1] *Institut Sophia Agrobiotech, Université de Nice-Sophia Antipolis, Sophia Antipolis, France frendo@unice.fr*
[2] *Institute for Integrative Biology of the Cell, CNRS, Gif-sur-Yvette, France*
[3] *Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR441, Castanet-Tolosan, France*
[4] *Interactions Arbres-Microorganismes, UMR1136, Université de Lorraine, Vandoeuvre-lès-Nancy, France*
[5] *Institut de Recherche en Horticulture et Semences, Université d'Angers, Angers, France*

Leguminous plants are associated with soil nitrogen-fixing bacteria to develop root nodules in which atmospheric nitrogen (N_2) is reduced to ammonium used by the host plant. In *Medicago truncatula*, the bacteria undergo a terminal differentiation into N_2-fixing bacteroids under the control of defensin-like nodule-specific cysteine-rich peptides (NCRs) produced by the host plant (1). The redox state of NCRs is important for their biological activity. Amongst the proteins involved in the regulation of redox state, thioredoxins (Trx) play key roles in the redox regulation of target proteins through the reduction of protein disulphide bonds. A nodule-specific Trx type called Trx s has been detected in *M. truncatula* (2). We showed that *M. truncatula* genome contained four *Trx s* amongst which *MtTrx s1* and *s3* were induced in the nodule infection zone. Analysis of the Trx s1 cellular localization in nodules using Trx s1::GFP translational fusion and immunolocalisation showed that Trx s1 is targeted to the symbiosome, the nitrogen-fixing organelle. Silencing of *Trx s1* using RNAi constructs reduces the nitrogen-fixation efficiency, reduced bacteroid size and impaired bacteroid DNA endoreduplication. The observation that Trx s1 is mainly expressed in nodule infection zone and localized in symbiosomes led us to postulate that NCRs may be Trx s1 substrates. Both NCR247 and NCR335 were found to interact with Trx s1 and we showed that Trx s1 expression enhanced the cytotoxic effect of NCR335 in *S. meliloti*. In conclusion, our results show that the plant partner modifies the redox state of NCR peptides and induces the bacterial terminal differentiation using specific thioredoxins.

References:

[1] *Mergaert et al. (2003) Plant Physiol. 132:161-173*
[2] *Alkhalfioui et al. (2008) Plant Physiol. 148:424-435*

POSTER 5B-4 /LIGHTNING TALK/

Genetic Dissection of Nodulation Signalling using the *LORE1* Insertion Mutant Collection

Anna Malolepszy, Terry Mun, Yasuyuki Kawaharada, Niels Sandal, Dorian F. Urbanski, Jens Stougaard and Stig Uggerhøj Andersen

Centre for Carbohydrate Recognition and Signalling, Aarhus University, Aarhus C, Denmark

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With more than 640,000 annotated insertions, the non-transgenic *LORE1* insertion mutant resource offers knock-out alleles of the majority of active genes in the legume *Lotus japonicus* (http://lotus.au.dk). A detailed characterization of the *LORE1* population, including insertion preferences, transcriptional effects of insertion and estimates of background mutation rates will be presented. In addition, examples of use of the *LORE1* resource in characterization of nodulation signalling will be presented.

References:

Urbański DF, Małolepszy A, Stougaard J, Andersen SU. (2012) Genome-wide LORE1 retrotransposon mutagenesis and high-throughput insertion detection in Lotus japonicus. Plant Journal vol. 69 (4) pp. 731-41.

Fukai E, Soyano T, Umehara Y, Nakayama S, Hirakawa H, Tabata S, Sato S, Hayashi M. (2012) Establishment of a Lotus japonicus gene tagging population using the exon-targeting endogenous retrotransposon LORE1. Plant Journal vol. 69 (4) pp. 720-740.

Urbański DF, Małolepszy A, Stougaard, J., and Andersen, S.U. (2013). High-Throughput and Targeted Genotyping of Lotus japonicus LORE1 Insertion Mutants. Methods in molecular biology 1069: 119-146.

Fukai, E., Malolepszy, A., Sandal, N., Hayashi, M. & Andersen, S.U. (2014) Forward and reverse genetics: The LORE1 retrotransposon insertion mutants. in The Lotus japonicus genome (eds. Tabata, S. & Stougaard, J.) 221-229, Springer.

POSTER 5B-5 /LIGHTNING TALK/

The Profile of NCR Peptides Produced by the Legume Host Correlates with the Morphotype of the Bacteroids

Jesús Montiel Gonzalez, Attila Szűcs, Éva Kondorosi and Attila Kereszt

Biological Research Center of the Hungarian Academy of Sciences. Szeged, Hungary

Within the nitrogen fixing root nodules, bacteroids can be reversibly or terminally differentiated depending on the legume host. Terminal differentiation is characterized by cell enlargement, genome amplification and loss of reproductive ability. *Medicago truncatula* and closely related species from the inverted repeat-lacking clade (IRLC) control terminal differentiation of bacteroids with the production of nodule-specific cysteine-rich (NCR) peptides (1). Recently, we characterized terminal differentiation of bacteroids in other six legumes that represent distinct genus from the IRLC (2). Interestingly, the degree of cell elongation was rather variable and resulted in different morphotypes: swollen (S), elongated (E), spherical (SP) and elongated-branched (EB). These findings let us to further explore bacteroid differentiation and NCR acquisition in ten IRLC legumes. For this purpose, nodule transcriptomes from six IRLC legumes were sequenced, and analyzed together with genome/transcriptome data available from another four IRLC species. *NCR* genes were identified in all of these species, however the number of *NCR* genes was considerably different, ranging from 6 NCRs in *Glycyrrhiza uralensis* up to several hundreds in *Medicago* species. A positive correlation was found between the number of the expressed *NCR*s and cell growth of the bacteroids. Legumes governing EB-bacteroid development (*Galega orientalis*, *Pisum sativum* and *Medicago* species) evolved the largest NCR families. In contrast, legumes with S-bacteroids have the lowest number of *NCR* genes. In addition, both the percentage and expression of cationic NCR were higher in legumes with EB-bacteroids compared to IRLC legumes accommodating S-, SP- or E-bacteroids. The analysis of the transcriptome and genome data from 10 species of the IRLC strongly suggests that NCR are directly implicated in cell-elongation of the bacteroids and particularly, the cationic NCR seems to be involved in the EB-morphotype. This notion is supported by the expression pattern of anionic and cationic NCR in the different zones of *M. truncatula* nodules. In zone I, 10% of the NCR transcripts are cationic, however in the interzone and nitrogen-fixing zone they reach 45% and 39%, respectively. These data provide further insights into the evolutionary process that led to NCR acquisition and terminal differentiation of the bacteroids. Several attempts are in progress to understand the emergence and diversification of NCR, as well as their role in shaping bacteroids morphotype.

References:

[1] *Kondorosi et al. (2013) Annu. Rev. Microbiol. 67:611-628.*
[2] *Montiel et al. (2016) MPMI. 29: 210-219.*

POSTER 5B-6 /LIGHTNING TALK/

Identification of Novel Symbiotic Plant Genes with the Help of *M. Truncatula Tnt1* Insertional Mutants

Szilárd Kovács, Ernö Kiss, Boglárka Oláh, Sándor Jenei, Erzsébet Fehér-Juhász and Gabriella Endre

Biological Research Centre, HAS, Institute of Genetics, Szeged, Hungary

A very efficient way of identifying important genes in model plants is the forward and reverse genetic analyses of mutants. The use of tagged mutant collections has already proved to be successful in revealing plant genes that function in the nitrogen-fixing symbiosis. In this work we have used the *Tnt1* insertion mutant collection of the model legume *M. truncatula* cv. Jemalong that was produced during the EU GLIP project (www.eugrainlegumes.org) in parallel to the one already existing at the Noble Foundation for ecotype R108 (http://bioinfo4.noble.org/mutant/). In the case of the GLIP collection, during the EU project only the construction of the mutant lines was financed. Consequently, only a limited number of mutants were characterized in this collection, despite the value of such characterized collections for the community. Now we have done a large scale symbiotic screen using this mutant collection and those lines were selected, in which individuals with impaired symbiotic phenotype appeared. We focused on those mutants that indicated defects in different steps of the symbiotic process, thus 24 lines were chosen for back-cross and further genetic analyses. From these lines segregating populations were produced for 11 mutants. In the meantime, FST sequences belonging to these lines were also carefully analyzed to use candidate gene approach. Thorough phenotype characterization and genotype determination of the candidate genes resulted in the identification of the mutated gene responsible for the symbiotic phenotype in two different mutant lines. The characterization of these two genes and their protein products is in progress and will be presented.

POSTER 5B-7 /LIGHTNING TALK/

What Defines and Regulates Nodule Identity and Organogenesis?

Katharina Schiessl, Jodi Lilley, Giles Oldroyd

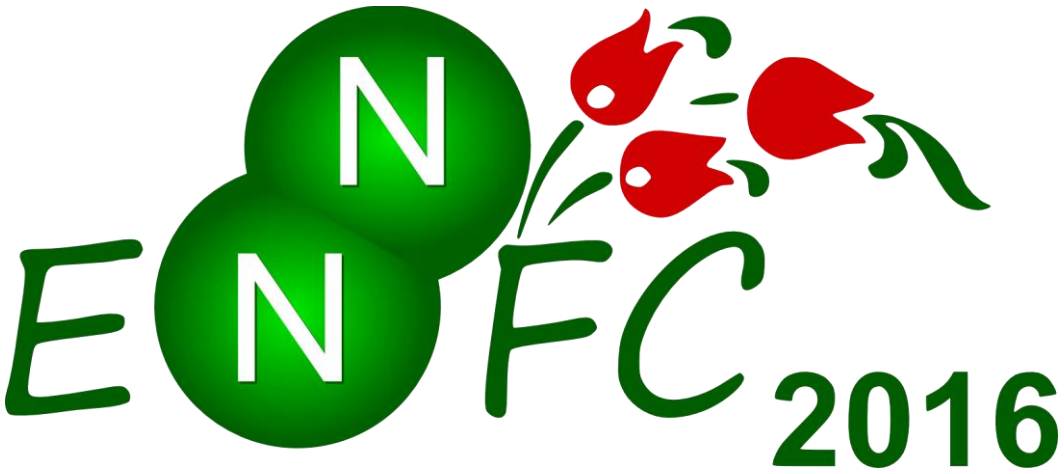
John Innes Centre, Colney Lane, Norwich, United Kingdom

Nodules are lateral organs that develop at the differentiation zone of legume roots in response to successful infection with symbiotic nitrogen fixing rhizobial bacteria. While studies on the symbiosis signalling pathway that leads to successful infection have identified many components of this pathway, little is known about the downstream gene regulatory networks that define nodule organ identity and direct nodule organogenesis.

Nodules emerged about 60 Mio years ago and are considered as a recent development in evolutionary terms (Sprent, 2001). Therefore, it has been hypothesised that nodules derived from lateral roots and have been modified in morphology and function to host the nitrogen fixing bacteria. Accordingly, it has recently been shown that several known key regulators of lateral root development such as members of the *PLETHORA* family play an important but modified role during nodule organogenesis (Franssen et al., 2015). However, there must also be novel genes that provide nodule identity and direct the unique development of these structures.

Here, we study nodule organogenesis in the model species *Medicago truncatula* which forms indeterminate nodules with a characteristic zonation consisting of an apical meristematic region, a transition zone where cells undergo endoreduplication, an infection zone and a nitrogen fixation zone in the central regions. In comparison to lateral root primordia that develop a central vasculature, nodules develop two or more vascular strands running along the periphery of the egg shaped nodule body, suggesting that vascular development plays a key role in nodule identity.

We aim to investigate the gene regulatory network that directs nodule identity and nodule organogenesis. In a first step, we used expression analysis with high spatial and temporal resolution on spot inoculated root sections and induced lateral roots. Comparative RNA-Seq experiments identified a set of differentially expressed genes between nodule and lateral root organogenesis over time course of development. This is be followed by detailed expression studies and functional analysis of key candidate genes. We hope that from these analyses we may be able to define the genes that provide the unique nodule identity, as well as better defining how lateral root developmental programmes underpin this process.



PARALLEL SESSION 7

Free-living Nitrogen Fixation

Chairs: Anton Hartmann, Rachel Foster

Synthetic Rebalancing of Nitrogen Fixation and Nitrogen Assimilation in Diazotrophs

Ana Bonnatto, Adam Gosztolai, Christopher Waite, Michal Komorowski, Volker Behrends, Mark Bennet, Michael Stumpf, Mauricio Baraona, Martin Buck, Jörg Schumacher

Division of Cell & Molecular Biology, Imperial College London, London, United Kingdom
j.schumacher@imperial.ac.uk

PII proteins play a central role in regulating the cellular nitrogen economy, perceiving the nitrogen status (glutamine/α-ketoglutarate ratio)(*1*), regulating adaptive responses of nitrogen assimilation and fixation in soil, and are also important during nodule development (*2*) (*3*). A low nitrogen status triggers post translational uridylylation of PII proteins by the uridylyl-transferase/removase (UT/UR, *glnD*). Most diazotrophs encode for both the canonical PII (*glnB*) and its highly homologous GlnK (*glnK*), which is generally co-transcribed with the ammonium transporter (*amtB).* In *E.coli*, PII and GlnK have overlapping functionalities in regulating both nitrogen assimilation gene expression (via NtrB/NtrC) and glutamine synthetase activity (GS), but GlnK may play more specific roles in ammonium transport and in diazotrophs in regulating nitrogen fixation via *nifL-nifA* systems. In contrast to the constitutively expressed *glnB*, *glnK* expression is generally subject to nitrogen status. A dynamic integrative systems understanding of the PII regulated nitrogen economy is currently lacking.

In order to gain a quantitative understanding of PII responsiveness to environmental changes in *E.coli,* we accurately determined the absolute intracellular concentrations of PII, GlnK and GS, as well as their post translational states, and in relation to changing glutamine and α-ketoglutarate levels, using MRM-MS targeted proteomics and NMR metabolomics, respectively. Our models show that both *glnB* and *glnK* are required for maximal growth under ammonium limiting conditions, explained by altered GS adenylylation kinetics and that the post translational states of PII and GlnK both depend on sequestration of GlnK (to AmtB) away from UT/UR. Broadly speaking, we found that PII is the major control point for NtrB/NtrC mediated transcription regulation, while both PII and GlnK can effectively act on GS enzyme activity, allowing for rapid adaptation to ammonium shock. When tracing the information flow through the coupled PII-GS post translational system we observed a perhaps unprecedented inherent information capacity, suggesting a fine metabolic tuning capacity of the central nitrogen assimilation pathway. We present ongoing work on (BB/N003608/1) how we exploit these insights to synthetically rewire cell signalling in diazotrophic *Klebsiella oxytoca*, rebalancing N-assimilation and N-fixation for surplus ammonium secretion.

References:

[1] *J. Schumacher et al., Nitrogen and carbon status are integrated at the transcriptional level by the nitrogen regulator NtrC in vivo. mBio 4, e00881 (2013).*

[2] *R. Dixon et al., Genetic regulation of biological nitrogen fixation. Nat Rev Microbiol 2, 621 (2004).*

[3] *T. Arcondeguy et al., The Rhizobium meliloti PII protein, which controls bacterial nitrogen metabolism, affects alfalfa nodule development. Genes & development 11, 1194 (1997).*

Using Synthetic Biology to Increase Nitrogenase Activity

Xin-Xin Li, Xiao-Meng Liu, Hao-Wen Shi and San-Feng Chen

State Key Laboratory for Agrobiotechnology and Biological Science College, China Agricultural University, Beijing, China

Nitrogen fixation has been established in protokaryotic model *Escherichia coli* by transferring a minimal *nif* gene cluster composed of 9 genes (*nifB, nifH, nifD, nifK, nifE, nifN, nifX, hesA and nifV*) from *Paenibacillus* sp. WLY78 (1). However, the nitrogenase activity in the recombinant *E. coli* 78-7 is only 10% of that observed in wild-type *Paenibacillus* (1-3). Thus, it is necessary to increase nitrogenase activity through synthetic biology.

In order to increase nitrogenase activity in heterologous host, a total of 28 selected genes from *Paenibacillus* sp.WLY78 and *Klebsiella oxytoca* were placed under the control of *Paenibacillus nif* promoter in two different vectors and then they are separately or combinationally transferred to the recombinant *E. coli* 78-7. Our results demonstrate that *Paenibacillus suf* operon (Fe-S cluster assembly) and the potential electron transport genes *pfoAB, fldA* and *fer* can increase nitrogenase activity. Also, *K. oxytoca nifSU* (Fe-S cluster assembly) and *nifFJ* (electron transport specific for nitrogenase) can increase nitrogenase activity. Especially, the combined assembly of the potential *Paenibacillus* electron transporter genes (*pfoABfldA*) with *K. oxytoca nifSU* recovers 50.1% of wild-type (*Paenibacillus*) activity. However, *K. oxytoca nifWZM* and *nifQ* can not increase activity.

References:

[1] *Wang et. al. (2013) PLoS Genet. 9:e1003865.*
[2] *Zhang L. and Chen S. (2015) World J Microbiol Biotechnol. 31:921–927.*
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Regulation of Alternative Nitrogenase Expression by σ54-Dependent Activator Homologs in *Azotobacter vinelandii*

Corinne Appia-Ayme and Ray Dixon

Department of Molecular Microbiology, John Innes Centre, Norwich Research Park, Norwich, United Kingdom

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Azotobacter vinelandii is one of the few diazotrophs that can express three different types of nitrogenase dependent upon metal availability, namely the conventional molybdenum enzyme (encoded by *nif* genes), the vanadium nitrogenase (encoded by the *vnf* gene cluster) and the iron-only nitrogenase (encoded by the *anf* gene cluster). In addition to the three specific activators, NifA, VnfA and AnfA, required for transcriptional activation of the molybdenum, vanadium and iron-only nitrogenase operons respectively, the *A.vinelandii* genome encodes an additional homolog of NifA, designated NifA2 and two homologs of VnfA, designated as VnfA2 and VnfA3. Each of these activator homologs has a regulatory GAF domain, which in the case of the VnfA homologs contains conserved cysteine residues thought to bind an iron-sulphur cluster. Furthermore the DNA binding recognition helices of these homologs are extremely similar to those of either NifA of VnfA, suggesting that the activator homologs have similar DNA-binding targets. We have been focusing on VnfA3, which exhibits 71 % identity with VnfA. Transcriptional profiling of *A. vinelandii* reveals that *vnfA3* expression is elevated in the presence of vanadium or in iron-only conditions (1). *VnfA3* is located in an operon with Avin_47110, which encodes a putative vanadate binding protein. We have found that transcription of this operon requires VnfA, and is likely to be activated from a σ54-dependent promoter with a putative VnfA binding site upstream. Experiments with a *vnfA3* deletion mutant suggest that VnfA3 is not required to activate transcription from either the *vnfH*, *vnfD* or *vnfE* promoters. However, VnfA3 is necessary to activate expression of the iron only (*anf*) system. A model to explain the complex hierarchy of metal-dependent gene regulation mediated by the VnfA homologs will be discussed in this talk.

References:

[1] *Hamilton, T. L. et al.(2011) Transcriptional Profiling of Nitrogen Fixation in Azotobacter vinelandii. Journal of Bacteriology 193, 4477-4486*

Macro-molecular Model Indicates Multiple Oxygen Management Strategies by *Crocosphaera watsonii*

Keisuke Inomura and Michael Follows

Massachusetts Institute of Technology, Cambridge, United States

Crocosphaera is one of the major nitrogen fixers in the ocean. To protect nitrogenase from oxygen, they perform oxygenic photosynthesis in the day and nitrogen fixation at night. However, even in the dark they must manage oxygen at a cost with ecological and biogeochemical impacts. Here we examine oxygen management of *Crocosphaera* by developing a quantitative "cell flux model", which couples a simplified metabolic network with cross-membrane transport, conserving mass, electron and energy flow at the individual scale. We use the model to examine the value of three oxygen management strategies: respiratory protection, size adaptation, and an Extracellular Polymeric Substance barrier to diffusion. Incorporating all of these improves simulations of lab culture studies, suggesting that *Crocosphaera* adopts these three oxygen management strategies.

POSTER 7-1 /LIGHTNING TALK/

Isolation and Characterization of Two New Nitrogen Fixing Unicellular Cyanobacteria from the Indian Ocean

Sofie Vonlanthen and Rachel A. Foster

Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, Sweden

In the marine environment, cyanobacteria are often considered the most important nitrogen fixing organisms, providing new bioavailable nitrogen to the oligotrophic oceans. Although larger filamentous and heterocyst forming symbiotic species, such as ***Trichodesmium*** and ***Richelia*** have previously been considered the dominant nitrogen fixers in the open ocean, more recent studies have found that unicellular cyanobacteria play an equally important role (1). The unicellular cyanobacteria group B (UCYN B), represented by the cultured strain ***Crocosphaera watsonii***, is widely distributed across tropical and subtropical oligotrophic oceans (2). Six strains have been isolated from the Pacific and the Atlantic Oceans and whole genome comparisons have shown surprisingly low genetic variation between strains, despite phenotypic variations (3). Other known unicellular cyanobacteria include group C, closely related to the costal and benthic species of Cyanothece (4). Isolated strains of Crocosphaera are limited to two oceans (Atlantic and Pacific) and to further investigate the diversity of unicellular nitrogen fixing cyanobacteria we aimed to isolate new strains, from the previously under sampled Indian Ocean.

Two new strains (SU2 and SU3) of unicellular cyanobacteria were isolated from the waters outside Zanzibar, Tanzania. Both strains have a coccoid or oval shape measuring 3-5µm and grow in medium lacking combined nitrogen.

Phylogenetic analysis of the 16srRNA and nifH genes show that one strain (SU2) is more closely related to C. watsonii (96% and 91%, respectively) and one (SU3) is more closely related to Cyanothece (99% and 98% respectively).

Both strains produce large amounts of extra cellular polysaccharides as imaged by Alcain blue staining and microscopy, and SU3 form large visible colonies comprised of hundreds of cells. Experiments to measure Carbon and Nitrogen fixation were performed with $^{15}N_2$ and ^{13}C-bicarbonate. Both bulk and single cell rates will be presented from analysis using secondary ion mass spectrometry (SIMS). In addition, whole genome sequencing was performed on both species using Illumina Miseq. The GC content of SU2 and SU3 is 40.1 and 36.6% respectively. SU2 and SU3 represent the first two isolated strains of diazotrophic unicellular cyanobacteria from Indian Ocean.

References:

[1] *Montoya et al. (2004) Nature 430:1027-32*
[2] *Moisander et al. (2010) Science 327:1512-14*
[3] *Bench et al. (2013) J Phycol 49:786-801*
[4] *Foster et al. (2007) Limnol. Oceanogr 52:517-32*

POSTER 7-2 /LIGHTNING TALK/

Molybdenum Metabolism in *Azotobacter vinelandii*

Mónica Navarro-Rodriguez, Emilio Jiménez-Vicente and Luis M. Rubio

Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid, Spain

Azotobacter vinelandii is a model organism for biochemical studies of N_2 fixation that has the peculiarity of being able to fix N_2 under aerobic conditions. This process could be carried out via three homologous nitrogenases that differ in the heterometal found at their active sites: the Mo nitrogenase, the V nitrogenase and the Fe-only nitrogenase (1). Expression of either Mo nitrogenase or the alternative nitrogenases depends on the metal availability in the medium, with molybdate acting as co-repressor of alternative nitrogenases. In addition, *A. vinelandii* carries a molybdenum storage protein, referred to as MoSto, which can store up to 25-fold more Mo than needed during maximum nitrogenase activity (2,3). In this work we investigate a plausible role of MoSto as an obligate intermediate in the Mo trafficking pathway that provides molybdenum for the biosynthesis of the iron-molybdenum cofactor (FeMo-co) of the Mo nitrogenase. We analyze the phenotype of an *A. vinelandii mosAB* in-frame deletion mutant strain, which lacks the genes encoding the MoSto subunits. Wild type and mutant strains are compared in: (*i*) their ability to grow diazotrophically and non-diazotrophically in media containing decreasing amounts of molybdate; (*ii*) their levels of intracellular Mo; (*iii*) the *in vivo* levels of Mo nitrogenase activity; (*iv*) their capacity to repress expression of the alternative nitrogenases.

References:

[1] *Jacobson, M. R., et al (1986). Journal of Bacteriology, 167(2), 480–6.*
[2] *Pienkos, P. T. and Brill, W. J. (1981). Journal of Bacteriology, 145(2), 743–51.*
[3] *Hernández JA, et al (2009) Biochemistry. 48(41):9711-9721.*

POSTER 7-3 /LIGHTNING TALK/

Studies of DraB, a Small Thioredoxin Like Protein in *Rhodospirillum rubrum* with an Unknown Function Encoded Within the *dra* Operon

S. Gudise, A. Bock, S. Rosowski, S. Nordlund, Agneta Norén

Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden

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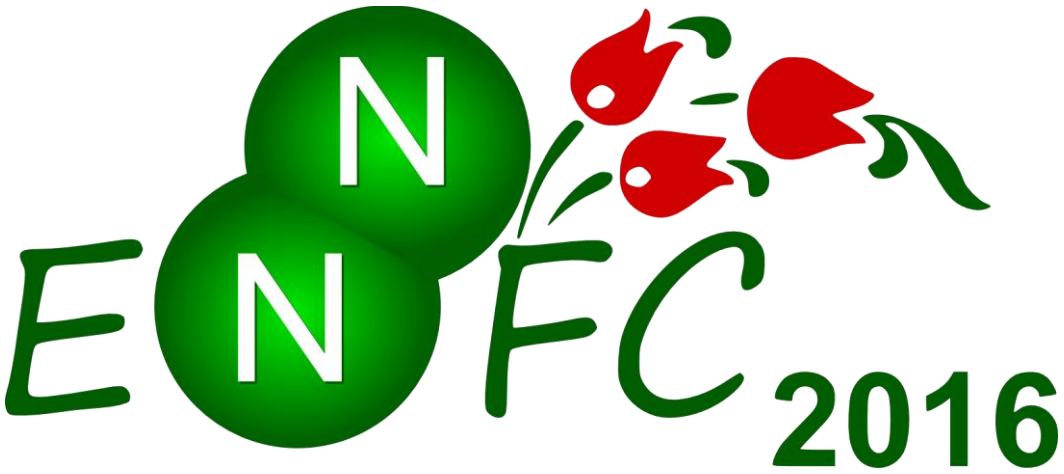
Nitrogen fixation is studied at the molecular level in the free-living diazotroph, *Rhodospirillum rubrum,* where nitrogenase activity is controlled at a post- translational level with signalling pathways transducing changes in nitrogen/energy status. The pathway for energy (and nitrogen) signalling includes 2 regulatory proteins DraT and DraG in our model organism. These proteins are involved in reversible ADP ribosylation of nitrogenase that regulates nitrogenase activity. We have earlier shown that this involves protein-protein interactions and changes in cellular localization of DraG as a response to high ammonia conditions. Presently the energy signalling pathway is our focus and we have shown that changes in energy supply do lead to association of DraG to the membrane in *R. rubrum*, both when the light is turned off in photoheterotrophically grown cells and when oxygen is removed in nitrogen-fixing cultures grown aerobically in the dark. In *R. rubrum* DraT and DraG are encoded in an operon where an additional gene, *draB,* encoding a protein with unknown function is present. The expression, purification and localization of DraB and construction of a *draB* mutant is in progress. The primary sequence of DraB reveals high similarity to proteins in the thioredoxin family thereby indicating a possible role in redox reactions. DraB is now to be characterized and further investigated for a possible role the regulation inresponse to cellular energy status. The redox changes in our model organism can be manipulated by light/darkness, oxygen concentration and growth on different carbon sources.

The methods used include basic DNA overexpression systems with tagged target proteins (FLAG, his, GST) for further purification and detection. Protein protein interactions will be investigated by immunoprecipitation. Localization studies include cell fractionation along with Western blot analysis. A *draB* mutant is constructed by the insertion of an antibiotic cassette in the gene and further transformed into an *Eschrichia coli* S-17 strain for conjugation with *R.rubrum* to create a *draB* mutant. In addition for elucidate DraB properties spectroscopy, crystallization and various biochemical methods are used.



PLENARY SESSION 8

On the Interface of Symbiotic/Pathogenic Interactions

Chair: Attila Kereszt

Role of Plant Innate Immunity in the Legume, Nitrogen Fixing Symbiosis

Gary Stacey

Divisions of Plant Science and Biochemistry, University of Missouri, Columbia, United States

The classical model for the evolution of symbiotic systems is that they begin as acute, pathogenic interactions and then evolve through adaptation and attenuation of virulence, ultimately reaching the state of a commensal or mutualistic interaction. If this model has relevance to the nitrogen-fixing legume symbiosis, it is somewhat surprising that only a modest amount of research has focused on the role of plant pathogen defense mechanisms in the establishment and maintenance of the symbiosis. Indeed, there is a long history of research that provides anecdotal support for a clear role for plant defense pathways. For example, papers showing that key plant defense hormones (e.g., salicylic acid, jasmonic acid, ethylene, etc.) significantly impact the nodulation process. However, it would appear that these papers have been largely viewed by the community as relating to ancillary functions and, perhaps, not central to, for example, the intensely studied Nod factor-signaling pathway crucial to nodulation. However, recent published results appear to signal a significant, although still modest, shift in the thinking about the role of innate immunity in the nodulation process. For example, it is now clear that type-three, protein secretion systems, classically involved in the delivery of pathogen effector proteins, can play essential roles in nodulation, especially in those cases where the Nod factor signaling pathway can be circumvented. Likewise, there is now evidence that the rhizobial symbiont can actively suppress host immunity and this, if not essential, does clearly promote nodulation. Thus, my reading of the community is that there is a new appreciation for the potential impact of plant immunity on symbiotic nitrogen fixation. However, many of the pertinent questions remain unanswered. Including, whether plant innate immunity plays a central role (i.e., essential for nodulation) or whether it can only be viewed as a modifier of host range and/or overall infection efficiency. There is also relatively little knowledge available with regard to the specific mechanisms by which nodulation and innate immunity pathways, both relatively well studied, intersect. Our laboratory is focusing on these types of questions and we will present our most recent findings that begin to provide some insight, including the mechanisms by which Nod factor signaling impacts plant innate immunity. Our results suggest a key role for well-known components of the plant immunity system in core plant functions involved in nodulation.

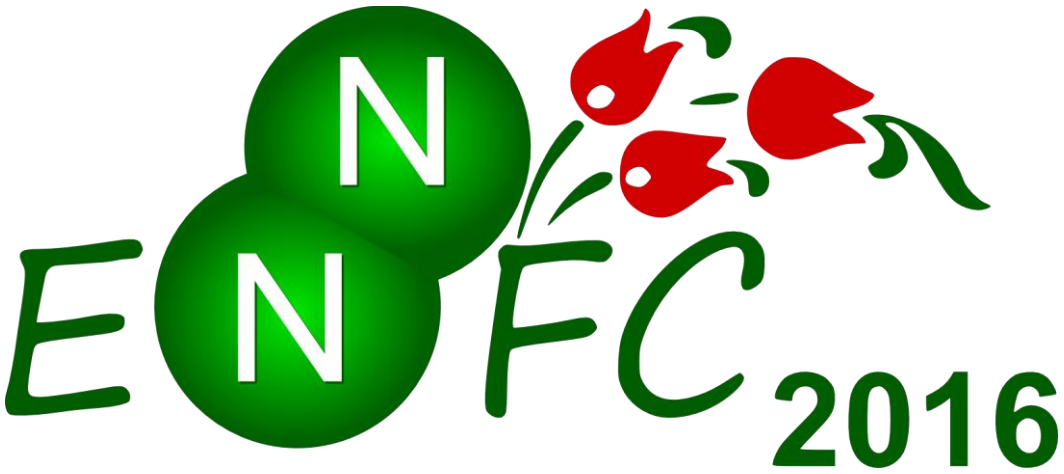
The *Medicago truncatula NAD1* Gene is Essential for the Persistence of Bacteroids in Symbiotic Nodules

Ágota Domonkos[1], Szilárd Kovács[2], Ernő Kiss[2], Anikó Gombár[1], Gyöngyi Kováts[1], Lili Fodor[1], Beatrix Horváth[1], Mónika Tóth[1], Ferhan Ayaydin[4], Károly Bóka[5], Attila Kereszt[3], Gabriella Endre[2], Péter Kaló[1]

[1] *NARIC, Agricultural Biotechnology Institute, Gödöllő, Hungary*
[2] *Institute of Genetics, Biological Research Centre, Szeged, Hungary*
[3] *Institute of Biochemistry, Biological Research Centre, Szeged, Hungary*
[4] *Cellular Imaging Laboratory, Biological Research Centre, Szeged, Hungary*
[5] *Department of Plant Anatomy, Eötvös Loránd University, Budapest, Hungary*

Legumes form root nodules to accommodate nitrogen-fixing soil bacteria termed rhizobia. Rhizobia invade nodule cells wherein they are hosted intracellularly in plant derived membrane compartments called symbiosomes. During the invasion of the nodules, wild type plants control their immune response and no visible marks of defense responses can be observed. In order to identify genes that control the plant immunity during the invasion of the symbiotic nodule, we analyzed the nodule sections of ineffective symbiotic mutants of *Medicago truncatula* for the presence of the hallmarks of induced defense responses, such as pigment accumulation or enhanced autofluorescence. Two mutants showing induced defense responses were identified in the deletion and insertion mutant collections of *M. truncatula* and the gene identification experiments revealed that the two lines carry new alleles of the recently cloned *nad1* mutant (Wang et al. 2016 New Phyt). Monitoring of the expression of pathogen-related genes revealed their increased transcript level confirming the induction of defense responses. The detailed phenotypic characterization of the mutants with the new alleles and the analysis of the expression pattern of the symbiosis specific and pathogen-related genes proved that *NAD1* functions in the control of the plant immune responses to maintain bacterial persistence in the symbiotic cells following the rhizobial invasion of the nodule.

Keywords: symbiotic nitrogen fixation, defense response, Medicago truncatula, rhizobia



PLENARY SESSION 9

Evolution, Diversity and Ecology

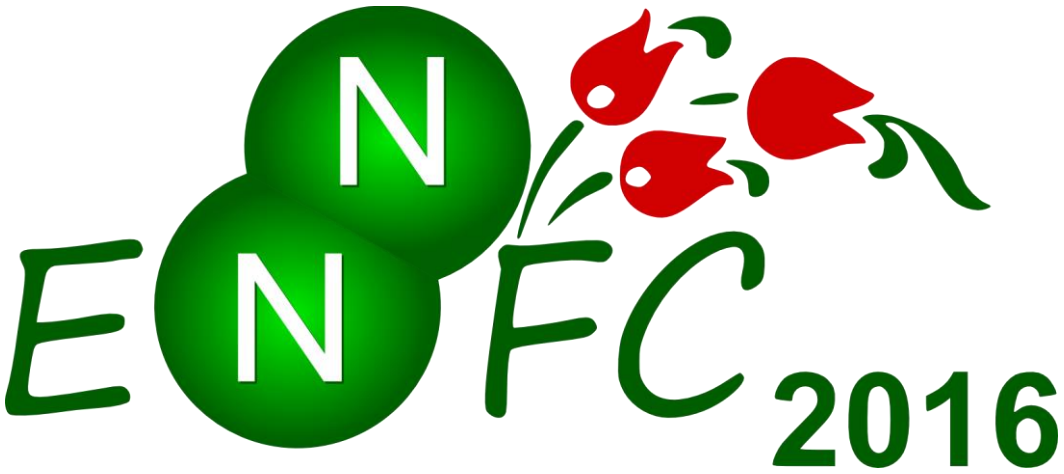
Chair: Attila Kereszt

Evolution of Symbioses: From Phylogeny to Intelligent Design

Pierre-Marc Delaux

Laboratoire de Recherche en Sciences Végétales, Université de Toulouse, CNRS, UPS, Castanet Tolosan, France

Evolution gave rise to extremely complex traits, such as intracellular symbioses, in specific plant lineages. Large engineering projects are now trying to re-create these traits in other species to improve crops productivity and the sustainability of our agricultural systems. However, a comprehensive understanding of the mechanisms governing these traits is required in order to engineer them. Phylogenetic and comparative phylogenomic approaches conducted on the wide spread arbuscular mycorrhizal symbiosis resulted in the identification of hundreds of candidate genes missed by conventional forward genetics. It is now tempting to speculate that similar approaches would strongly improve our understanding of the root nodule symbioses. This would bring the basic knowledge to engineer them in so far non-nodulating species. Progresses made by an international consortium in that direction will be presented.



PARALLEL SESSION 8

On the Interface of Symbiotic/Pathogenic Interactions

Chairs: Rene Geurts, Pascal Ratet

Symbiotic Roles of the Type III Secretion System in *Bradyrhizobium elkanii*

Shin Okazaki, Hiroki Miwa, Michiko Yasuda, Sachiko Masuda

Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan

Root-nodule symbiosis between leguminous plants and nitrogen-fixing bacteria (rhizobia) involves molecular communication between the two partners. Key components for the establishment of symbiosis are rhizobium-derived lipochitooligosaccharides (Nod factors, NFs) and their leguminous receptors (NFRs) that initiate nodule development and bacterial entry.

Previously, we demonstrated that symbiosis in the soybean rhizobium Bradyrhizobium elkanii is promoted by the type III secretion system (T3SS), which delivers virulence factors via pathogenic bacteria. Intriguingly, T3SS of *B. elkanii* activates host nodulation signaling in the absence of NFs and NFRs. In contrast, T3SS of *B. elkanii* causes nodulation restriction on soybeans carrying *Rj4* allele.

We have identified several T3SS-related genes in *B. elkanii* that affect symbiotic interactions with host legumes. Functional properties and symbiotic roles of the novel T3SS-related genes will be discussed.

Exploring the Immune Status of Nodules

Fathi Berrabah[1,2], Jeoffrey George[1,2], Thierry Bailleau[3], Michel Zivy[3], Fabienne Vailleau[4], Pascal Ratet[1,2], Benjamin Gourion[1,2,4]

[1] *Institute of Plant Sciences Paris Saclay IPS2, CNRS, INRA, Université Paris-Sud, Université Evry, Université Paris-Saclay, Orsay, France*
[2] *Institute of Plant Sciences Paris-Saclay IPS2, Paris Diderot, Sorbonne Paris-Cité, Orsay, France*
[3] *Plateforme d'Analyse Protéomique de Paris Sud-Ouest, Unité Mixte de Recherche de Génétique Végétale, Ferme du Moulon, Paris, France*
[4] *Laboratoire des Interactions Plantes Microorganismes, Université de Toulouse, INRA, CNRS, Castanet Tolosan, France*

In the legume nodules, the density of the rhizobial population is higher than during many pathogenic interactions but the plants do not develop visible defense reactions (1). How these plants tolerate the massive and chronic invasion by rhizobia remains poorly understood. The aim of our studies is to better understand the molecular basis of the legume tolerance to rhizobia and whether this has any consequences in term of vulnerability towards pathogens. A hypothesis is that immunity is specifically suppressed in the symbiotic organ and that it might be deleterious for plant health in the presence of pathogenic organisms.

Using genetics, proteomics, molecular biology, pharmaceutical approach as well as microscopy, we showed that repression of the plant ethylene signaling pathway plays a key role in the tolerance to intracellular rhizobia (2). Furthermore, a patho-symbio-system was set up in order to further characterize the immune status of the nodules and investigate the consequence of nodulation on plant vulnerability to pathogens. The nodules sensitivity to infection, its capacity to support the growth of an armed or disarmed pathogen and its capacity/incapacity to spread the pathogen in the rest of the plant when it is the initial infected site are explored. Results will be presented and discussed.

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The Incompatible Interaction between *Medicago truncatula* A17 and *Sinorhizobium meliloti* RM41 Induces Early Nodule Senesce

Péter Körmöczi[1], Kata Terecskei[1], Anikó Gombár[3], Edit Ábrahám[1], Andrea Borbola[2], Ting Wang[1], Lili Fodor[3], Attila Szűcs[1], Éva Kondorosi[1], Péter Kaló[3], Attila Kereszt[1]

[1] *Institute of Biochemistry, Biological Research Centre HAS, Szeged, Hungary*
[2] *Institute of Genetics, Biological Research Centre HAS, Szeged, Hungary*
[3] *Agricultural Biotechnology Institute, NARIC, Gödöllő, Hungary*

Medicago truncatula establishes nitrogen-fixing symbiosis with *Sinorhizobium* species *S. meliloti* and *S. medicae*. To identify the genetic determinants of the symbiotic process large-scale mutagenesis programs have been initiated in both partners using chemical mutagens, fast-neutron bombardment or transposable elements (1). These programs have helped to isolate a number of genes that are essential for the initiation and development of the symbiotic process. By exploring the natural variations existing among different ecotypes and strains of the plant and bacterial partners, respectively, one can identify genes/proteins that are required only under certain conditions, with certain partners and/or participate in the fine-tuning of the interaction (2-4).

The *M. truncatula* A17 ecotype that has a sequenced genome can establish effective symbiosis with a number of *S. meliloti* and *S. medicae* strains including the reference strain 1021, but it is ineffective with strain RM41. *M. truncatula* DZA315.16 and A20 ecotypes, however, form effective symbiosis with both bacteria. We have shown with microscopic analysis that although bacteriod development occurs in the incompatible nodules, final nodule differentiation is arrested and bacteroids that are unable to fix nitrogen disappear from the nodule cells that are repopulated by saprophytic bacteria.

We compared the transcriptome of 10 and 21 day old nodules formed on the roots of two M. truncatula ecotypes (A17 and DZA315.16) after inoculation with Rm41 to identify those bacterial and plant pathways that are differentially expressed in the effective and incompatible interactions. We could detect the early and elevated expression of senescence related genes (cysteine proteases, chitinases and phosphatases). In addition, several genes involved in defense response (resistance proteins) showed altered expression in the incompatible nodules. The increased expression of about 40 transport mechanism related bacterial genes in the inefficient nodules was also observed.

To identify the bacterial gene responsible for the trait a genomic library from an effective bacteria has been introduced into strain Rm41 and transposon mutagenesis of the ineffective bacterium has been performed. The isolation of the plant gene has been initiated by map-based cloning.

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The Medicago *api* Gene is Required for Full Colonisation by *P. palmivora* as well as Nitrogen Fixing Bacteria

Aleksandr Gavrin[1], Thomas Rey[1], Abhishek Chatterjee[1], Justine Toulotte[1], Frédéric Debellé[2,3], Etienne Pascal Journet[3], Fernanda de Carvalho-Niebel[2,3], Hiroki Takagi[4], Ryohei Terauchi[4], Thomas Torode[1], Siobhan Braybrook[1] and Sebastian Schornack[1]

[1] *Sainsbury Laboratory (SLCU), University of Cambridge, Bateman Street, Cambridge, United Kingdom*
[2] *INRA, Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR441, Castanet-Tolosan, France*
[3] *CNRS, Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR2594, Castanet-Tolosan, France*
[4] *Iwate Biotechnology Research Center, Kitakami, Japan*

Medicago truncatula roots engage in diverse microbial interactions such as beneficial *Rhizobium*–legume symbiosis and infections by the oomycete pathogen *Phytophthora palmivora*. While both types of interactions seem to be quite different, they may in part rely on the same plant processes to be successful. Infection processes of Rhizobia and *P. palmivora* feature host intracellular stages. *Medicago* mutants impaired in colonisation by both types of microbes can help identifying the contributing plant genes.

We screened *M. truncatula* mutants affected in nitrogen fixing symbiosis for their resistance to *P. palmivora*. Seedlings of *api* (altered primordia invasion) mutants of *M. truncatula,* defective in nitrogen fixing symbiosis, also displayed reduced levels of *P. palmivora* penetration and disease progression. We employed genetic, transcriptomic, histological and biochemical approaches to identify the *API* gene and the mechanisms underlying the *api* phenotype. Our experiments suggest that *api* plants have a modified cell wall configuration leading to colonisation impairment by Rhizobia and *P. palmivora*.

POSTER 8-1 /LIGHTNING TALK/

Symbiosis or Defense: The Molecular Mechanism Involving LysM Receptors of the Model Legume *Lotus japonicus*

Zoltán Bozsóki[1], Jeryl Cheng[1], Mickael Blaise[2], Jens Stougaard[1], Simona Radutoiu[1]

[1] *Centre for Carbohydrate Recognition and Signalling (CARB), Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark*
[2] *Centre d'études d'agents Pathogènes et Biotechnologies pour la Santé, CNRS, Université de Montpellier, Montpellier, France*
zoltanbo@mbg.au.dk

Chitin oligomers derived from fungal cell walls are recognized as PAMPSs and elicit defense responses in plants. On the other hand symbiotic nitrogen fixing rhizobia bacteria produce signaling molecules with a chitin backbone that are essential for the symbiosis initiation (Nod factors). LysM motif containing receptors have been shown previously to be essential for perception of chitin-backbone-containing molecules. Rice CEBiP and Arabidopsis CERK1 are indispensable for chitin elicited defense signaling, while legumes mutated in *L. japonicus* NFR1 and NFR5 are unable to respond to Nod factors and symbiotic bacteria. We have identified and characterized the chitin receptor in *L. japonicus*, LYS6. *lys6* mutant plants fail to respond to chitin oligomers with oxidative burst, MAPK3/6 phosphorylation or defense gene activation, but are able to form efficient symbiosis with rhizobia and arbuscular mycorrhiza. On the other hand, *nfr1* mutants respond normally to chitin oligomers. NFR1 and LYS6 share 78% similarity in their amino acid sequences, are expressed in the same type of root cells, yet drive signaling pathways leading to opposing outcomes. We aim to identify molecular patterns present in the two receptors responsible for the symbiosis/defense decision at the very first steps of microbe recognition to broaden our understanding on the function and evolution of LysM receptor-mediated signaling in root cells. For this we use single and double mutant phenotypic analyses coupled with genetic complementation studies using synthetic receptor molecules. The results of our analyses will be presented and discussed.

POSTER 8-2 /LIGHTNING TALK/

Assessing the Relevance of a Range of Polysaccharide Signaling Molecules for Activation of Symbiotic Signaling

Jongho Sun[1], Feng Feng[1], Sarah Shailes[1], Simona Radutoiu[2], Jens Stougaard[2] and Giles Oldroyd[1]

[1] *John Innes Centre, Norwich, United Kingdom*
[2] *Department of Molecular Biology and Genetics, Centre for Carbohydrate Recognition and Signalling, Aarhus University, Denmark*

Plants associate with micro-organisms to facilitate nutrient acquisition, with most plants interacting with AM fungi, but only a few plants, such as legumes, associating with nitrogen-fixing rhizobial bacteria. Both AM fungi and rhizobia signal to the plant via diffusible lipochitooligosaccharides (LCOs) or chitooligosaccharides (COs) signaling molecules that can activate the common symbiosis signaling pathway with induction of calcium oscillations. Activation of symbiosis signaling by LCOs is mostly restricted to legumes, while perception of COs during symbiosis appears to occur more broadly within the plant kingdom. Recently, we have found that long-chain chitin oligomers CO8 can trigger not only ROS production and MAPK phosphorylation but also symbiotic calcium oscillations. In contrast, CO4 activates symbiotic calcium oscillations, but not PAMP responses. Both CO4 and CO8 activate symbiotic calcium oscillations in a range of plant species. These results indicate that CO8 is involved in both symbiotic and immune signaling, while CO4 appears to be limited to symbiotic signaling. Reverse genetic screening in both *Lotus japonicus* and *Medicago truncatula* revealed multiple LysM-RLK mutants with defects in CO8-induced PAMP and CO8/CO4 induced symbiosis signaling. These chitin receptors are closely related to, but different to the Nod factor receptors associated with perception of rhizobia. Our results suggest a complex of LysM-RLKs in legume roots that function in chitin perception and a separate complex of LysM-RLKs associated with Nod factor perception. While both complexes appear to function in symbiosis signaling, only the CO receptor complex also activates PAMP signaling.

POSTER 8-3 /LIGHTNING TALK/

The Investigation of the Mechanisms by which Pea Plants Discriminate and Respond to Structurally Related COs Signals from Symbiotic and Pathogenic Fungi

Irina Leppyanen, Nadezhda Vishnevskaya, Oksana Shtark, Vlada Shakhnazarova, Elena Dolgikh

All-Russia Research Institute for Agricultural Microbiology (ARRIAM), Saint Petersburg, Pushkin, Russia

Plants are able to effectively detect long-chain chitooligosaccharides (COs) released from the cell walls of pathogenic fungi that leads to defence reactions and inactivation of the potential pathogen (1-2). Structurally related short-chain COs (n = 4, 5), produced by mycorrhizal fungi, elicit symbiotic responses in plant roots leading to the symbiosis development (3). How plants perceive the structurally related CO signaling molecules to elicit defence and symbiotic responses respectively is the major objective of our work. Recently the LysM-containing receptor-like protein CERK1 was shown to have dual functions in symbiosis and immunity signaling and involved in COs perception in rice (4). To find out how pea plants *Pisum sativum* L. are able to distinguish COs signaling molecules with similar structure, the searching of CERK1-like receptor has been performed. As a result, the pea LysM-receptor kinase LYK9 was shown to be required for response to treatment with COs having various degree of polymerization. Treatment of pea plants with COs (n = 8-10) resulted in increased level of *PsLyk9* gene expression together with activation of defense genes markers (*PsPAL1*, *PsPAL2* and *PsPR10*). In addition, plants with suppression of *PsLyk9* gene expression using RNA interference were more susceptible to infection with low pathogenic fungus *Fusarium culmorum* (Wm.G.Sm.) Sacc. 891. Reduced disease resistance correlated with decreasing of genes expression encoding defense proteins and enzymes (*PsPR10*, *PsPAL2*). Moreover, in plants with repressed of *PsLyk9* expression treated by short-chain COs (n=5) a significant decrease in the expression of marker genes of arbuscular mycorrhizal symbiosis (*DELLA*, *NSP2*, *DRP* and *RAM1*) has been observed. These results allow us to conclude that the *Ps*LYK9 is the most likely CERK1 receptor homolog in pea that is involved in the control of plant immunity and AM symbiosis formation making the complexes with various co-receptors.

This work was supported by the Russian Scientific Foundation (RSF project no. 16-16-10043).

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POSTER 8-4 /LIGHTNING TALK/

Hopanoids Play an Important Role in *Bradyrhizobium* Strains during their Free-living and Symbiotic States

Nicolas Busset[1], A. Silipo[2], G. Kulkarni[3], D. Gully[1], C. Chaintreuil[1], J. Fardoux[1], D. Gargani[4], F. Gressent[1], A. Molinaro[2], D. K. Newman[3], E. Giraud[1]

[1] *IRD, UMR LSTM, Montpellier, France*
[2] *Dipartimento di ScienzeChimiche, Università di Napoli Federico II, Naples, Italy*
[3] *Division of Biology and Biological Engineering, CIT, Pasadena, United States*
[4] *CIRAD, UMR BGPI, Montpellier, France*

Unlike other rhizobia, the outer membrane (OM) of *Bradyrhizobium* strains contains pentacyclic triterpenoids called hopanoids, which can represent up to 40% of total lipids (1). These molecules, which display structural similarity with eukaryotic sterols, constitute an important class of membrane lipids widely distributed in diverse bacteria. Hopanoids are thought to act as membrane condensers, thus increasing bacterial resistance to various abiotic stresses. Interestingly, two classes of hopanoids are present in the OM of *Bradyrhizobium* strains, classical "free" hopanoids such as diploptene, diplopterol or tetrahymanol and a novel class corresponding to a C_{35} hopanoid molecule called bacteriohopanetetrol which is covalently linked to the lipid A and named HoLA (2). Here we investigate the role of hopanoids by mutating some genes involved in hopanoids biosynthesis in two *Bradyrhizobium* strains, the photosynthetic strain BTAi1 and the non-photosynthetic strain *B. diazoefficiens*.

In the strain BTAi1, a complete hopanoid deficient mutant was built by mutating the squalene hopene cyclase gene (*shc*) which is a key enzyme involved in the cyclisation of the squalene into hopanoid. Analysis shows that the mutant is more sensitive to various stresses under free-living conditions. On *Aeschynomene* plants, the *shc* mutant induces functional nodules but with a life span drastically reduced indicating an important role of hopanoids for maintaining chronic intracellular infection (2).

In the strain *B. diazoefficiens,* we were unable to obtain an *shc* mutant. This suggests that hopanoids synthesis is essential for growth and survival of *B. diazoefficiens*. However, we succeeded to obtain two other mutants in the two most important hopanoids family, 2-methylated (2Me) and extended (C_{35}) hopanoids by deleting respectively the *hpnP* and *hpnH* genes (3). Our results indicate that both classes of hopanoids play an important role in free-living state under various stress conditions. When the mutants were tested on plant, no effect of the *hpnP* mutation was observed, while the *hpnH* mutant was found to be altered for several aspects of the symbiosis, including evasion of plant defense reactions, or nitrogen fixation in symbiosis with *A. afraspera* but not with soybean. The difference observed between the two hosts for the *hpnH* mutant is likely related to the presence of cysteine-rich antimicrobial peptides in *Aeschynomene* nodules that induce drastic modification in bacterial physiology (3).

In conclusion, hopanoids play an important role to optimize bacterial survival in both free-living and symbiotic states of *Bradyrhizobium* strains.

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POSTER 8-5 /LIGHTNING TALK/

Unraveling Plant Cellular Targets for the *Rhizobium*-specific Effectors NopL and NopP

I. Jiménez-Guerrero[1], F. Pérez-Montaño[1], C. Medina[2], E. Santero[2], J. A. Monreal[3], F. J. Ollero[1] and Francisco J. López-Baena[1]

[1] *Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla. Sevilla, Spain*
[2] *Departamento de Biología Molecular e Ingeniería Bioquímica, CABD, Universidad Pablo de Olavide/CSIC/Junta de Andalucía, Sevilla, Spain*
[3] *Departamento de Fisiología Vegetal, Facultad de Biología, Universidad de Sevilla. Sevilla, Spain*

Pathogenic Gram-negative use a specialized apparatus called Type 3 secretion system (T3SS) to deliver effectors directly into the eukaryotic host cells. These effectors suppress plant defenses to promote disease but they can also be recognized by specific plant receptors that trigger a strong defense reaction to eliminate the pathogen (1). The T3SS has also been found in some symbiotic rhizobial strains and the effectors secreted are involved in host-range determination and symbiotic efficiency.

The broad host-range bacterium *S. fredii* HH103 secretes two proteins through the T3SS, NopL and NopP, which are specific for rhizobia. In this work we studied the function of both effectors in the symbiosis with soybean, which is considered its natural host plant. NopL and NopP were phosphorylated by soybean root kinases and the phosphorylation cascade was Ca^{2+}- and calmodulin-dependent. While the signaling pathway that culminates in the phosphorylation of NopL included ser/thr and MAPKK kinases, in the case of NopP this pathway involved ser/thr and tyr kinases but not MAPKK kinases.

Transient expression of both *nopL* and *nopP* fused to YFP in *Nicotiana benthamiana* leaves and further confocal imaging indicated that they localized to the nucleus of the host cell. The use of a yeast-based array to determine possible effectors functions indicated that NopP could be involved in nuclear localization and migration. Finally, co-immunoprecipitation analyses of *N. benthamiana* NopL- and NopP-interacting proteins and further mass spectrometry analyses identified several potential plant targets for these effectors. The most interesting interactions are currently being validated by Bimolecfular Fluorescence Complementation (BiFC)

This work was supported by project P11-CVI-7050 of the Junta de Andalucía.

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POSTER 8-6 /LIGHTNING TALK/

Specialised Protein Secretion in Plant-Microbe Symbioses

Onur Oztas[1], Huairong Pan[1], Minsoo Kim[1], Christina Stonoha[1], Xiaoyi Wu[2], Bin Wang[2] and Dong Wang[1]

[1] *University of Massachusetts, Amherst, MA, United States*
[2] *Nanjing University, Nanjing, China*

Legumes can engage rhizobia, transforming free-living bacteria into nitrogen-fixing organelles (symbiosomes). This symbiosis co-opts the machinery of a more widespread association with arbuscular mycorrhizal (AM) fungi[1]. Both symbionts are outlined by a specialized interfacial membrane, which is derived from the host plasma membrane but maintains its unique identity. The protein secretory pathway has been co-opted to deliver host factors to the microsymbiont[2,3]. We recently discovered that the fidelity of this symbiosis-specific protein secretion is insured by a t-SNARE protein generated by a transcriptional regulatory mechanism called alternative cleavage and polyadenylation, and that this symbiotic t-SNARE is crucial to a properly developed symbiosis[4].

Unlike AM fungi, rhizobia exist intracellularly through a persistent infection. Sustaining and controlling such a chronic infection is a major challenge to the host, and could be a key factor in the emergence of the nitrogen-fixing symbiosis. We discovered that to insure the survival of newly internalized rhizobia, the host processes certain symbiosome membrane proteins to suppress excessive defence responses. As bacteroids mature, a new set of factors is needed to maintain the viability of such enlarged bacteria. We discovered that one of the Nodule-specific Cysteine-Rich (NCR) peptides is required for the survival of differentiated bacteroids[5]. Current evidence suggests this NCR peptide localizes to the bacterial surface, and could promote bacteroid survival by facilitating metabolite exchange between the host and the bacteria.

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POSTER 8-7 /LIGHTNING TALK/

Rhizobia Inoculation Reduces *Didymella pinodes* Impacts on Photosynthetic Efficiency of *Pisum sativum*

Getinet Desalegn[1], R. Turetschek[2], H.-P. Kaul[1], S. Wienkoop[2]

[1] *Department of Crop Sciences, University of Natural Resources and Life Sciences, Tulln, Austria*
[2] *Department of Ecogenomics and Systems Biology, University of Vienna, Vienna, Austria*

The needs for sustainable crop production systems and plant-based protein for both human and animal consumption are rising globally. In these aspects, field pea (*Pisum sativum* L.) often plays an important role due to its ability to fix atmospheric nitrogen and high protein content. It can also form symbiotic association with **arbuscular mycorrhiza (AM) fungi which facilitate plant uptake and transport of relatively immobile soil nutrients such as phosphorus and others.** However, the global expansion of *P. sativum* production has major abiotic and/or biotic impediments, of which *Didymella pinodes* is a very damaging foliar disease (1, 2) through reduction of photosynthetic efficiency of pea plants. Elsewhere up to 70% yield losses of peas caused by this pathogen have been reported. On the other hand, recent evidences showed that a type of root-associated microbes that increase plant growth and yield, elicit induced systemic resistance against pathogens (3, 4, 5). We evaluated the effects of below ground rhizobia and AM fungi **inoculations on photosynthetic efficiency and production of dry matter of two field pea genotypes (cultivar Messire and Protecta). We found that** there were significant differences between some of the group averages of pea plants **in green area, leaf greenness, photosynthetic efficiency and shoot dry matter production. T**he highest pathogen infection and lowest photosynthetic components such as leaf greenness and green areas were observed in susceptible cultivar Messire in which about **50% green area reduction was found as compared to healthy plants. Under pathogen attack, the rhizobia increased more dry matter production than AM fungi by 23%.** Furthermore, the lowest pathogen infections and highest photosynthetic efficiency results were recorded with cultivar Protecta inoculated with *Rhizobium*.

Overall, we conclude that the belowground rhizobial and AM fungi symbiotic associations enhance significantly photosynthetic efficiencies and production of shoot biomass compared to the control treatment in both *P. sativum* cultivars under *D. pinodes* attacks.

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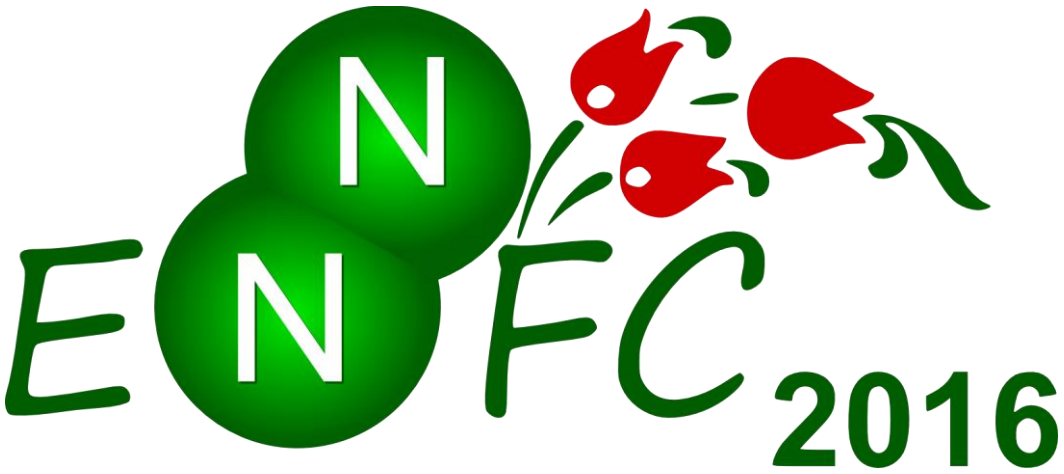

PARALLEL SESSION 9

Evolution, Diversity and Ecology

Chairs: Euan James, Peter Young

Genetic Dissection of the Rhizobium Nodulation Trait using Interspecific Crosses between Symbiotic *Parasponia* and Non-symbiotic *Trema* Species

Arjan van Zeijl, F. Bu, R. Holmer, R. van Velzen, E. Fedorova, W. Kohlen, T. Bisseling, R. Geurts

Department of Plant Sciences, Laboratory of Molecular Biology, Wageningen University, Wageningen, The Netherlands

Nitrogen-fixing rhizobium symbiosis has evolved in parallel in two lineages; namely legumes and Parasponia. The Parasponia-rhizobium symbiosis is relatively young when compared to legumes, as phylogenetic studies indicated that the Parasponia lineage only recently emerged from an ancestral Trema species (1). To facilitate studies into the evolution of rhizobium symbiosis, we established a comparative platform for Parasponia-Trema research. This platform includes germplasm of several Parasponia and Trema species, genome assemblies and protocols for *in vitro* propagation and stable transformation. As Parasponia and Trema are closely related we questioned whether interspecific hybrids could be generated. Such hybrids will provide a powerful tool to genetically dissect the rhizobium nodulation trait. Among the six combinations tested, we found one combination that can give rise to viable F1 hybrid plants; namely the cross between diploid *P. andersonii* and tetraploid *T. tomentosa*. Analysis of these F1 hybrid plants genetically separated nodulation from intracellular accommodation of rhizobium. As hybrid plants were able to form nodules we conclude that nodulation is a genetically dominant trait. In contrast, intracellular infection is a recessive trait as hybrid nodules do not form infection threads, and subsequently do not fix nitrogen. Conceptually there are three possible scenarios that can lead to a dominant genetic trait; (I) missense mutation(s) resulting in dominant alleles, (II) mutations causing -directly or indirectly- significant elevated expression of genes or (III) the gain of new genes. Experiments using the calcium reporter cameleon indicated that Parasponia, but not Trema, is able to respond to rhizobium Nod factors with calcium spiking (2). Based on this, we hypothesize that the dominant character conferring nodulation ability is expressed in Parasponia roots already prior to inoculation. To study whether scenario II. and/or III. apply for the dominant character of nodulation, we performed a comparative transcriptomic analysis on non-inoculated root segments of the elongation-differentiation zone of two *Parasponia* and three *Trema* species, as well as on F1 hybrid plants. To this end, we took advantage of the genome sequences that we have generated for Parasponia and Trema and the identification of orthology pairs for most Parasponia and Trema genes. These studies indicate that the root transcriptomes of Parasponia and Trema are quite distinct. Pathway analysis suggests that several pathways involved in hormone homeostasis are differentially expressed between Parasponia and Trema species, which is supported by direct quantifications of hormone concentrations. In current experiments we focus on validation of candidate pathways in symbiosis using reverse genetic analyses.

References:

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Root Nodule symbiosis in *Lotus japonicus* Drives the Establishment of Distinctive Rhizosphere, Root, and Nodule Bacterial Communities

Rafal Zgadzaj[1]*, Ruben Garrido-Oter[1]*, Dorthe B. Jensen[2], Paul Schulze-Lefert[1] and Simona Radutoiu[2]

[1] *Department of Plant-Microbe Interactions, Max Planck Institute for Plant Breeding Research, Cologne, Germany*
[2] *Department of Molecular Biology and Genetics, Aarhus University, Denmark*

* *co-first authors*

The legume *Lotus japonicus* has been employed for decades as a model to study the establishment of binary symbiotic relationships with nitrogen-fixing rhizobia that trigger organogenesis of root nodules for bacterial accommodation. Using community profiling of 16S rRNA gene amplicons we reveal that the wild-type *L. japonicus* distinctive nodule and root communities are established by parallel rather than consecutive selection of a wide taxonomic range of bacteria from the rhizosphere and root compartments. Comparative analyses of *Lotus* wild-type and symbiotic mutant plants *nfr5*, *nin1* and *lhk1*, identified a previously unsuspected role of the nodulation pathway in the establishment of distinctive bacterial assemblages in root and rhizosphere. We found that the loss of nitrogen-fixing symbiosis dramatically alters community structure in the root and rhizosphere, affecting at least 14 bacterial orders. Our findings imply a role of the legume host in selecting a broad taxonomic range of root-associated bacteria that, in addition to nitrogen-fixing rhizobia, increases plant growth and ecological performance.

Molecular Evolution of Paralogous Symbiotic Receptor Kinase Genes in Pea (*Pisum sativum* L.)

Vladimir A. Zhukov[1], Anton S. Sulima[1], Alexander I. Zhernakov[1], Liudmila A. Lutova[2], Igor A. Tikhonovich[1,2]

[1] *All-Russia Research Institute for Agricultural Microbiology, St.-Petersburg, Russia*
[2] *Saint-Petersburg State University, Faculty of Biology, St.-Petersburg, Russia*

Specificity of interactions between legume plants and nodule bacteria is based on ligand-receptor interactions, during which the bacterial signal molecules (Nod factors) are perceived by plant receptor kinases. Corresponding genes have been cloned more than 10 years ago in *Lotus japonicus* (*NFR1* and *NFR5*) and *Medicago truncatula* (*LYK3*) (1, 2, 3). In pea, 2 paralogous genes encoding receptor kinases (*Sym37* and *K1*) are known as homologs of *NFR1* of *L. japonicus* and *LYK3* of *M. truncatula*. These genes are located in cluster on LG I of pea, and mutations in one of these genes (*Sym37*) block symbiosis (4). Also, the gene *Sym2*, that is located at the same cluster, was described more than 30 years ago as a determinant of host specificity (probably encoding receptor kinase recognizing the Nod factor structure). Screening of pea genome BAC library (in collaboration with Dr. Helene Berges, CNRGV, Toulouse, France) led to identification of the third paralogous gene encoding receptor kinase that is clustered together with *Sym37* and *K1*. This gene, named *LykX*, is considered as the most promising *Sym2* candidate.

In this work, the fragments of 1st exons of the pea genes *Sym37*, *K1* and *LykX* encoding the receptor parts of the corresponding proteins were sequenced and analyzed. Using the set of 99 pea genotypes that represent virtually all the diversity within the *Pisum* genus, the information about the extent of polymorphism of these gene parts was obtained. Based on the analysis of DNA sequences using methods of molecular evolution it was shown that different genes were subjected to different types of selection pressure. The sequence of one of the receptor genes (*LykX*) contains the site which seems to be critical for the functioning of the encoded protein; this site was found to be under the significant negative selection pressure. The *K1* gene sequence possesses multiple polymorphic sites, which means that the positive selection acts in favor of several allelic states of the gene. The *Sym37* gene sequence, according to the McDonald-Kreitman test, underwent the pressure of positive (directional) selection.

The assumption that these paralogous genes were differentiated for realization of the diverse functions is reinforced by the results obtained. In general, the data obtained provide insights on the evolution and functioning of symbiotic systems formed by leguminous plants.

This work was supported by the Russian Science Foundation [grants number 14-24-00135 and 16-16-00118] and the Russian Foundation for Basic Research [grant number 15-29-02737].

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POSTER 9-1 /LIGHTNING TALK/

Stress-induced DNA Double-strand Break NHEJ Repair in *Sinorhizobium meliloti*: A Function in Lateral Gene Transfer?

Pierre Dupuy, Laurent Sauviac, Claude Bruand

LIPM, Université de Toulouse, INRA, CNRS, Castanet-Tolosan, France

DNA double-strand breaks (DSBs) are a permanent threat for living organisms, as they are a source of genome instability. Inefficient DSBs repair can lead to disorders ranging from reduced survival in bacteria to the development of cancers in humans. DSBs are repaired by several systems, including non-homologous end-joining (NHEJ). In eukaryotes, the main NHEJ proteins, Ku70 and Ku80, bind DNA ends as a heterodimer, and then recruit several additional proteins including enzymes which catalyze the modification and ligation of DNA ends. NHEJ has also been characterized in a limited number of bacteria, where the repair mechanism appears to be less complex than in eukaryotes. Indeed, only two proteins are required: a homodimeric Ku protein, and a multifunctional LigD enzyme which modifies and ligates the DNA ends. However, most studies were performed on bacterial species encoding a single ku-ligD pair. Actually, many bacterial species encode multiple copies of these genes, whose relative contributions to NHEJ in vivo are so far unknown.

The Sinorhizobium meliloti genome encodes four putative Ku (ku1-4) and four putative LigD (ligD1-4). To date, a single study conducted on this model bacterium showed that every ku single mutant is more sensitive than the wild type strain to ionizing radiations (1) showing that all ku genes are involved in NHEJ repair of DSBs in this organism.

Here, using a plasmid circularization assay, we performed a comprehensive genetic characterization of NHEJ repair in S. meliloti, and clarified the respective contributions of the various ku and ligD genes. We also demonstrated that NHEJ repair is activated under various stress conditions, including heat and nutrient starvation, and that part of this repair is under the control of the general stress response regulator RpoE2. Finally, for the first time in bacteria, we provided evidence that NHEJ not only repairs DSBs, but can also erroneously integrate heterologous DNA molecules into the breaks.

Altogether, our data provide new insights into the mechanisms of DSB repair in bacteria which encode multiple NHEJ systems, but also suggest that NHEJ might contribute to the evolution of bacterial genomes under adverse environmental conditions by participating in the acquisition of foreign DNA from distantly related organisms during horizontal gene transfer events.

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POSTER 9-2 /LIGHTNING TALK/

Mixed Nodules in *Sinorhizobium meliloti* - *Medicago sativa* Symbiosis Suggest the Presence of a Cheating Behavior

Alice Checcucci[1], Elisa Azzarello[2], Marco Bazzicalupo[1], Marco Galardini[3], Alessandra Lagomarsino[4], Stefano Mancuso[2], Lucia Marti[2], Maria C. Marzano[2], Stefano Mocali[4], Andrea Squartini[5], Marina Zanardo[5], Alessio Mengoni[1]

[1] *Department of Biology, University of Florence, Florence, Italy*
[2] *Department of Agri-food Production and Environmental Science (DISPAA), University of Florence, Florence, Italy*
[3] *EMBL-EBI, Wellcome Trust Genome Campus, Cambridge, United Kingdom*
[4] *Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Centro di Ricerca per l'Agrobiologia e la Pedologia (CREA-ABP), Firenze, Italy*
[5] *Department of Agronomy, Food, Natural Resources, Animals and the Environment, University of Padua, Legnaro, Padova, Italy*

In the symbiosis between rhizobia and leguminous plants host plants can form symbiotic root nodules with multiple rhizobial strains, potentially showing different symbiotic performances in nitrogen fixation. Here, we investigated the presence of mixed nodules, containing rhizobia with different levels of mutualisms, and evaluate their relative fitness in the *Sinorhizobium meliloti* - *Medicago sativa* model symbiosis.

We used three *S. meliloti* strains, the mutualist strains Rm1021 and BL225C and the non-mutualist one AK83. We performed competition experiments involving both *in vitro* and *in vivo* symbiotic assays with *M. sativa* host plants.

We show the occurrence of a high number (from 27% to 100%) of mixed nodules with no negative effect on both nitrogen fixation and plant growth. The estimation of the relative fitness as non-mutualist/mutualist ratios in single nodules shows that in some nodules the non-mutualist strain efficiently colonized root nodules along with the mutualist ones.

In conclusion, we can support the hypothesis that in *S. meliloti* – *M. sativa* symbiosis mixed nodules are formed and allow non-mutualist or less-mutualist bacterial partners to be less or not sanctioned by the host plant, hence allowing a potential form of cheating behavior to be present in the nitrogen-fixing symbiosis.

POSTER 9-3 /LIGHTNING TALK/

Structure and Functional Design of the Plasmid Regions Harboring *sym* Genes in *Rhizobium leguminosarum*: New Evidence for Intensification of Horizontal Gene Transfer and Narrowing the Host Range in Rhizobia Evolution

Elizaveta R. Chirak, V. V. Kopat, A. K. Kimeklis, E. E. Andronov, N. A. Provorov

ARRIAM, St-Petersburg, Russia

Our research is aimed to elucidate the trade-off between the structure of *sym* gene clusters in the evolutionary advanced rhizobia (*Rhizobium leguminosarum*), their host specificity and the impact of horizontal gene transfer (HGT) in the rhizobia evolution.

We investigated the structure of plasmid loci containing *sym* genes (*nod*-, *nif*- and *fix*-operons) in *Rhizobium leguminosarum* strain Vaf12 obtained from nodules of *Vavilovia formosa* (the relict legume, close to a common ancestor of the *Fabeae* tribe) in comparison to 7 strains of *R. leguminosarum* differing in origin and host specificity, including 4 strains of biovar *viciae* - symbionts of pea (TOM, UPM1131, 3841) and forage beans (248), as well as 3 strains of biovar *trifolii* - clover symbionts (WSM1689, WSM1325, SRDI943). We shown that the strains of *R. leguminosarum* bv. *viciae*, having *nod*X gene (controls acetylation of Nod-factor that determines the ability of rhizobia to establish symbiosis with broad spectrum of host plants, including the "Afghan" pea lines homozygous for sym^{2A} allele) are characterized by a less compact clustering of *sym*-genes than strains lacking *nod*X. In strains with *nod*X, symbiotic cluster size is 94.5±3.5 kb, while the proportion of *sym*-genes in this cluster is 36.5±1.5%. For the strains without *nod*X these indices are 61.7±3.7 kb and 56.3±1.4%, respectively (significant difference at P_0 <0.01). No correlation between the *sym* gene cluster size and the presence of *nodX* was found in biovar *trifolii* strains for which *nodX* is not involved in the host specificity control.

We detected the synthenic structures of *sym* genes in strains Vaf12, UPM1131 and TOM, and synthenic structures of areas located between *sym* genes in strains Vaf12, TOM and WSM1689. The correlation coefficients between the matrices of genetic distances in the analyzed strains for *nod*ABC, *nif*HDK, *fix*ABC operons, reach 0,993±0,002, while for plasmid sites located between *sym*-genes the correlations are substantially less (0.706±0.010). In these inter-*sym*-gene areas, 21-27% of genes are involved in the transport and metabolism of amino acids, which is substantially greater than the average for the genome of *R. leguminosarum* bv. *viciae* (11-12%).

These data suggest that the evolution of *R. leguminosarum* bv. *viciae*, defined by a narrowing of the host range (associated with the loss of *nod*X), was accompanied by a reduction of the *Sym* plasmid areas, located between *sym*-genes, as well as by specialization of these areas to perform functions related to symbiotic N_2 fixation. The observed increase of *sym*-gene density may be associated with the intensification of HGT in the populations of rhizobia, which determines the speed of evolution for the symbiotic gene system.

POSTER 9-4 /LIGHTNING TALK/

The Range of Rhizobia in New Zealand Soils

Mitchell Andrews

Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln, Christchurch, New Zealand

Most legume species can obtain a substantial amount of their nitrogen (N) requirements for growth from symbiotic bacteria (general term rhizobia) in root nodules and this can give them an advantage in low N soils if other factors are favourable for growth. There are only four genera of native legumes on the main New Zealand islands. These are *Carmichaelia, Clianthus, Montigena* and *Sophora*. However, over the past 150 years, several legumes have become important crop plants in New Zealand agricultural systems. Also, over 100 legume species from different continents have become naturalised in New Zealand and several of these are now important weeds.

Recent work which genotypically characterised rhizobia of native, crop and weed legumes in New Zealand and examined their cross-nodulation ability, was reviewed. The New Zealand native legumes were exclusively effectively nodulated by novel strains of *Mesorhizobium* which did not nodulate crop or weed legumes (1, 2). Seven groups of these strains have been formally described as new species (3, 4). Clovers (*Trifolium* spp.), lucerne (*Medicago sativa*), *Lotus* spp. and grain legumes were effectively nodulated by different genera, species and biovars of rhizobia primarily originating from inoculum. Weed legumes were effectively nodulated by different genera and species of rhizobia depending on plant species (5, 6). Novel strains of *Bradyrhizobium* that cross-nodulate lupins (*Lupinus* spp.), gorse (*Ulex europeus*), European broom (*Cytisus scoparius*) and tagasaste (*Cytisus proliferus*) are widespread in New Zealand.

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POSTER 9-5 /LIGHTNING TALK/

Genetic Evidence that Local Legume Sanctions Drive the Emergence of Symbiotic Nitrogen Fixation

Delphine Capela, Benoit Daubech, Ginaini Doin de Moura, Marta Marchetti, Catherine Boivin-Masson

LIPM, Université de Toulouse, INRA, CNRS, Castanet-Tolosan, France

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The selective forces allowing the emergence and maintenance of nitrogen-fixing mutualism between legumes and rhizobia are still highly debated. The most widely accepted explanation for the evolutionary persistence of mutualism is that plants differently affect the fitness of rhizobia in nodules based of their nitrogen-fixing abilities. This "sanction" hypothesis has been demonstrated by manipulating N2 gas abundance around nodules, simulating rhizobia with different fixation efficiencies. A genetic reappraisal of this question was needed to definitely confirm the sanction hypothesis and identify experimental conditions favoring the fixation of emerging Fix+ mutations within a non fixing bacterial population.

To evaluate whether, when and how plants differently treat fixing and non-fixing symbionts, we inoculated *Mimosa pudica* with either its wild-type symbiont *C. taiwanensis* or its isogenic *nifH* deletion mutant, or with a mix of both strains, and monitored the reproductive fate and bacteroid persistence of nodule bacteria along time.

We confirmed that plant sanctions non-fixing bacteria at the nodule level. Experimental evolution showed that legume sanctions drive the fixation of emerging nitrogen-fixing symbionts within a non-fixer population.

POSTER 9-6 /LIGHTNING TALK/

Symbiotic Divergence of *Rhizobium leguminosarum* Strains from Relict Legume *Vavilovia formosa*: A Background for Identification of Novel Biovar

Anastasiia K. Kimeklis[1], V. I. Safronova[1], A. A. Belimov[1], E. E. Andronov[1], I. G. Kuznetsova[1], A. L. Sazanova[1], A. R. Pukhaev[2], K. P. Popov[3], J. A. Akopian[4], N.A. Provorov[1]

[1] *All-Russia Research Institute for Agricultural Microbiology, St.-Petersburg, Russia*
[2] *Gorsky State Agrarian University, Vladikavkaz, Russia*
[3] *North-Ossetian Nature Reserve, Republic North-Ossetia-Alania, Alagir, Russia*
[4] *Institute of Botany of National Academy of Sciences of Republic Armenia, Yerevan, Republic of Armenia*

Tribe *Fabeae* (syn. *Vicieae*) comprises 5 genera, and typical rhizobial symbiont of plants from this tribe is *Rhizobium leguminosarum* bv. *viciae* (1). Symbiosis between this genus of rhizobia and plants of *Fabeae* tribe playing a major part in agriculture ecosystems, it's specifity is thoroughly studied (2). Exception is relict wild-growing *Vavilovia*, whose genus consists of single species *Vavilovia formosa*. It is believed, according to both comparative morphology and classical genetics, to be the closest relative to the extinct common ancestor of tribe *Fabeae* (3). Study of *Vavilovia*'s symbiosis is complicated by its low distribution of populations and specific inaccessible environment of the high-mountain Caucasian region.

In this study we undertook expeditions to North Ossetia, Dagestan and Armenia, where populations of *Vavilovia* were found and several plants with nodules were collected.

For the genotypic characterization of 20 *Rhizobium*-related isolates full gene of 16S rDNA and symbiotic genes from *nod* and *nif* operons were sequenced. On the basis of chromosomal background, phylogenetic analysis showed that isolated strains are the closest to *Rhizobium leguminosarum* bv. *viciae*. They formed several clusters, which didn't correlate with geographic distribution. Phylogenetic study of symbiotic background demonstrates that isolates form separate cluster inside *R. leguminosarum* bv. *viciae* clade. It was also revealed that all 20 *R. leguminosarum* strains are carriers of *nod*X gene, encoding O-acetyl transferase, a nod-factor decorator, which is essential for forming of symbiosis with *Pisum sativum* cv. *Afghanistan*. All strains fall into one group on the basis of *nod*X sequence.

Sterile tube-test experiments with strains representing different geographic regions were conducted using three host plants – *P. sativum* SGE, *P. sativum* cv. *Afghanistan* and *Vavilovia formosa*. These tests demonstrated that isolates formed nodules on all plants, although nodules had different ability to fix nitrogen. Nitrogen-fixing activity was detected mostly only in symbiosis with *Vavilovia formosa* plants, although some strains formed nod^+fix^+ phenotype on *P. sativum* cv. *Afghanistan*.

Genome sequencing of the "true" Vavilovia symbiotic isolate Vaf12 demonstrated one notable feature: the spacious arrangement of sym-genes. Broad host-range strains Vaf12 and TOM, both of which contain *nod*X, have more extended sym-regions than strains without *nod*X. It hints, that loss of some sym-genes (nodX, in particular) and compaction of sym-region may be the stages in process of host specificity constriction in *R.leguminosarum*.

This work is supported by RSF grant 14-26-00094.

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POSTER 9-7 /LIGHTNING TALK/

The Impact of Host Genotype and Geographical Origin on *Rhizobium leguminosarum* Genetic Diversity

Sara Moeskjær[1], Asger Bachmann[1], Bjarni Vilhjalmsson[2], Luc Janns[1], Mikkel H. Schierup[2], J. Peter W. Young[3], Stig U. Andersen[1]

[1] *Department for Molecular Biology and Genetics, Aarhus Universitet, Denmark*
[2] *Bioinformatics Research Center, Aarhus University, Denmark*
[3] *University of York, York, United Kingdom*

The aim of the NCHAIN project (http://mbg.au.dk/nchain) is to increase the yield of organic clover fields by analyzing the genetics of both clover plants and their rhizobium symbionts. To initiate the study of rhizobium genomics, 249 rhizobium strains were isolated from white clover nodules from plants grown in France (74), the United Kingdom (33), and Denmark (142). For each site the clover varieties are known, and representative soil samples were collected and sent for chemical analysis. Furthermore site-specific GIS data was collected based on the site coordinates.

The genetic relatedness of the rhizobium strains was assessed through analysis of phylogenetic trees. The core genes *recA* and *rpoB*, are clearly divided into different clades corresponding to genospecies of *R. leguminosarum* as previously described (1). In contrast, phylogenies of the symbiosis genes *nodA* and *nodD*, located on the pRL10 plasmid, were grouped primarily according to geographic origin. To elaborate on these findings we are carrying out a Quantitative Qualitative Amplicon Diversity (QQAD) analysis on nodule (n>100) and soil samples from each of the 170 sampled sites to study the diversity of rhizobia within and between each site. Furthermore, comparisons of soil and nodule populations will reveal the effects of selection by the clover host.

References:

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POSTER 9-8 /LIGHTNING TALK/

Evolution of *fixNOQP* Genes Encoding for the High-affinity Cytochromoxidase: Insight from the Genomes of Symbionts from the Relic Legume *Vavilovia formosa*

Vladimir V. Kopat, E. E. Andronov, N. A. Provorov

ARRIAM, St-Petersburg, Russia

Recently, our Caucasian expedition obtained the relict legume plant *Vavilovia formosa* with well sampled roots and intact root nodules, suitable for rhizobia isolation (the first successful isolation). It is curious, that *Vavilovia formosa* as the closest living relative of the last common ancestor of the *Fabeae* tribe (1) can be considered as a valuable source of information about rhizobia evolution. Specifically, broad variety of microorganisms including *Rhizoboium, Tardiphaga* and *Bosea* was isolated from the nodules of vavilovia (2). The genome sequencing of these isolates and close inspection of this data led us to the earliest events of nodule bacteria evolution (*sym*-gene acquisition and *sym*-cluster formation).

In the context of evolution signs in rhizobia genomes, *Tardiphaga* Vaf07 is the most interesting strain (taxonomically close to *Rhodopseudomonas*). There are *fixABGHISKNOPQ* genes into *fixAB*, *fixGHSNOQPK* and *fixISNOQP* clusters, but lack of crucial *nod* and *nif* genes in the Vaf07 genome (that is the reason of inability to form nodules under gnotobiotic conditions). In spate of lacking *nod* genes, *Tardiphaga* is regularly isolated from nodules of Vavilovia and some other legumes, possibly due to co-infection with *nod*-harboring strains (3). As can be seen, *Tardiphaga* has two copies of *fixNOQP* cluster, coding a high affinity cbb3-type cytochrome c oxidase.

The most intriguing issue about these two copies is their deep phylogenetic divergence: the first copy belongs to the *Bradyrhizobiaceae* cluster while the second one belongs to the *Sinorhizobium-Rhizobium* cluster. According with our suggestion, the first step of rhizobium-legume symbiosis formation was acquisition of *fix* genes by rhizobia, so it is reasonable to evaluate significance of *fix* genes in the rhizobia evolution.

After a detailed analysis of *fixNOQP* genes polymorphism among different rhizobia and strains from diverse groups of *Proteobacteria* we concluded that evolution of these genes includes at least two duplications with subsequent divergence of copies. *Tardiphaga* Vaf07 seems to be the closest relative of ancestor strain in which the first duplication occurred. Considering, that the proposed ancestor of *Rhizobium* (*Agrobacterium*) doesn't have *fix* genes at all, it can be assumed that tardiphaga's ancestor was an origin of *fix* genes for *Rhizobium* and the transfer of these genes was occurred under conditions close to vavilovia symbiosis. The revealed *fixNOQP* polymorphism may reflect to adaptation of the *Rhizobiales* to various anaerobic niches occupied in soil and in plant tissues.

This finding highlights the interplay of vertical transmission and horizontal gene transfer in the rhizobia evolution and the importance of functional diversification of different copies of *fix* genes in diversification of the modern rhizobia species.

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[2] *Safronova V.I. et al., 2014. Antonie Van Leeuwenhoek. Vol. 105. P. 389-399.*

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POSTER 9-9 /LIGHTNING TALK/

Carbon Utilisation by Strains of *Rhizobium* spp. in Sterile Soil

Kathryn Wigley[1], Steve Wakelin[2], Derrick Moot[1], Hayley Ridgway[1]

[1] *Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln, Canterbury, New Zealand*
[2] *AgResearch Ltd, Lincoln Research Centre, Lincoln, Canterbury, New Zealand*

In New Zealand, the bacterium *Rhizobium leguminosarum* bv. *trifolii* strain TA1 is used to commercially inoculate white clover seed. Recently, the need for inoculating white clover in New Zealand has been questioned. This is due to the inability of TA1 to deliver plant growth benefits because it cannot compete with high titres of naturalised rhizobia in the soil (1). However, naturalised strains have variable symbiotic potential compared with TA1 ranging from 0 – 170% (1). Effective naturalised strains adapted to New Zealand soils could be the key to improving commercial inoculants which are greater than 60 years old (2).

Rhizobium strains that show promise *in vitro*, often fail to perform in the field. A critical reason is lack of understanding of the interactions of the isolates within the soil environment (3). γ MicroResp™ is a novel modification of the 96-well based MicoResp™ system (4) which uses γ-irradiated soil. It allows the measurement of a microorganism's ability to utilize common C sources released in rhizosphere exudates within a physical soil background. This provides fundamental information on a strains free-living saprophytic ability.

For this study, 19 diverse rhizobia strains sourced from an international collection and 9 strains recovered from soils in Canterbury, New Zealand, were tested for their ability to utilise 14 carbon sources. The carbon sources were predominantly sugars and amino acids commonly found in the rhizosphere.

The international strains of rhizobia formed 9 distinct phenotypic groups (p<0.05) and the New Zealand strains formed four distinct phenotypic groups (p<0.05) based on differences in soil C-utilization. Variation in carbon utilization among the 19 international strains could not be attributed to geographic origin. In both the international and New Zealand collections, some groups of strains utilised a wider variety of carbon compounds to a greater degree compared with strains in other groups. The ability to use a broad range of C sources provides information about the ability of a strain to exist saprophytically in the rhizosphere (5). This knowledge will aid in improved selection and deployment of "environmentally fit" commercial inoculants.

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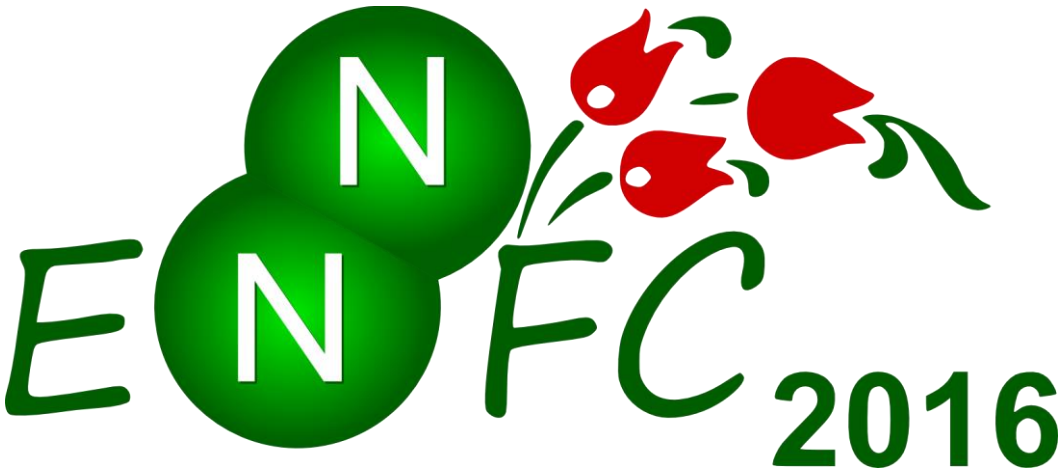


PLENARY SESSION 10

Commonalities and Specialities of Symbiotic Interactions

Chair:

Widespread Use of Antimicrobial Peptides in Bacterial Symbioses

Peter Mergaert[1,2], Tsubasa Ohbayashi[2,3], Shuji Shigenobu[4] and Yoshitomo Kikuchi[2,3]

[1] *Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Université Paris-Sud, Université Paris-Saclay, Gif-sur-Yvette, France*
[2] *Graduate School of Agriculture, Hokkaido University, Sapporo, Japan*
[3] *Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Hokkaido Center, Sapporo, Japan*
[4] *National Institute for Basic Biology (NIBB), Okazaki, Japan*
peter.mergaert@i2bc.paris-saclay.fr

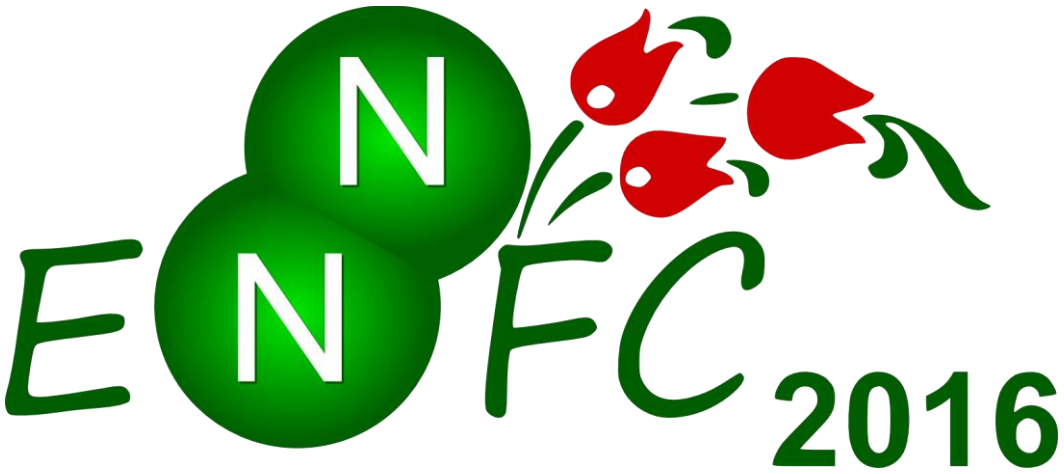
Symbiotic interactions with bacteria are common in eukaryotes and they usually have important functions in the biology of the host organism. The legume-rhizobium interaction is certainly one of the best understood symbioses and mechanisms that govern this interaction are described in great detail. Among the more recent insights in this symbiosis is the recognition that antimicrobial peptides (AMPs), called NCRs, are massively produced by the nodule cells, controlling the differentiation of the endosymbiotic rhizobium bacteria into bacteroids. By surveying evolutionary distant symbiotic systems in animals, plants and protists, we propose that symbiotic AMPs are universal and ancient mediators of bacterium-eukaryote symbiotic interactions. We will present our ongoing work on the symbiotic AMPs produced in two different insect-bacterium interactions. Close to 100 different Crypt-specific Cysteine-Rich peptides or CCRs are produced by the symbiotic organ of the bean bug *Riptortus pedestris*. This organ, located in the posterior region of the midgut is composed of crypts which are colonized by a single symbiont, a specific *Burkholderia* species. We propose that the CCRs contribute to the specific colonization of the crypts by the symbiotic *Burkholderia*. The pea aphid *Acyrthosiphon pisum* harbours the obligatory *Buchnera aphidicola* endosymbionts in specific cells called bacteriocytes located in the bacteriome organ. The bacteriocytes produce Bacteriocyte-specific Cysteine-Rich peptides or BCRs which have antimicrobial activity and target the *Buchnera*. We will argue that BCRs are possibly involved in the metabolic integration of the bacterial symbiont and the insect host.

The Diverse Bacterial Side of Lichens: Key to a New Concept of Symbioses

Martin Grube

Institute of Plant Sciences, Karl-Franzens-University, Graz, Austria

Lichens are well known for their diverse morphologies and their high tolerance to extreme habitats. In such environments, lichens are important contributors to carbon and nitrogen cycles. Evolutionary adaptivity and ecological significance of lichens is mediated by their symbiotic nature, which now turns out to be more complex than previously thought. Most biology textbooks present lichens still as the classic dual partnerships of fungi and algae (including cyanobacteria). However, in addition to their main algal partner, certain lichens harbor cyanobacteria in specialized organs, and recent high-throughput amplicon sequencing data show associations of lichens with diverse bacterial communities. Fluorescence *in situ* hybridization and confocal laser scanning microscopy specifically visualize the patterns of these communities on the surfaces and within the lichen structures. The comparison of omics approaches suggests that bacteria contribute to the lichen symbiosis, e.g. by nutrient supply, especially nitrogen, phosphorous and sulfur, resistance against biotic stress factors (pathogen defense), resistance against abiotic factors, support by production of vitamins, detoxification of metabolites, and lytic functions. Bacterial data (including those of cyanobacteria) can now be integrated in a comprehensive model of the symbiosis as a functional multi-player network of the participants. Thereby the classic view of two-tier symbiotic relationship can be extended to a more open view of symbioses, comprising tightly controlled relationships with one or few main players, as well as more fuzzy relationships with an additional set of microbial partners.



PLENARY SESSION 11

The Present and the Future Agricultural Use of BNF

Chair:

Dissecting and Engineering Symbiosis Signalling

Giles E. D. Oldroyd

Department of Cell and Developmental Biology, John Innes Centre, Norwich Research Park, Norwich, UK

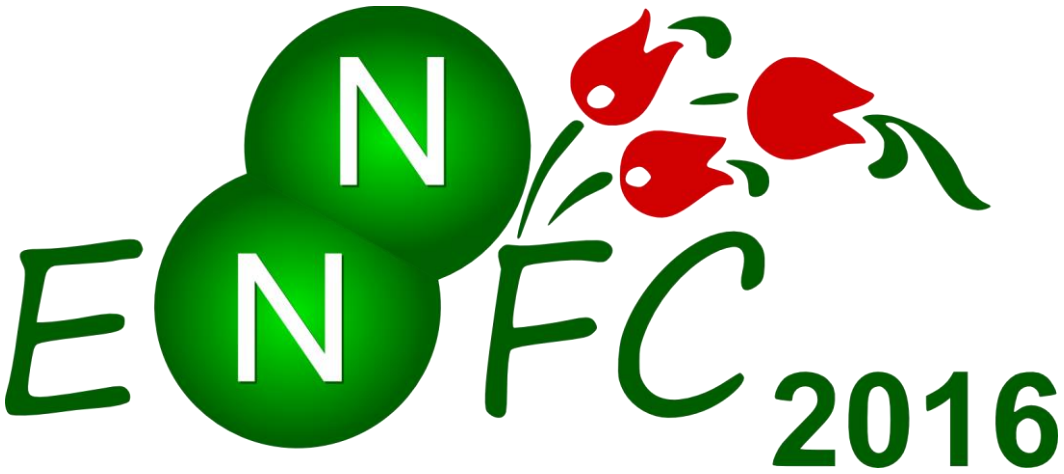
The ability to take up mineral nutrients, particularly nitrogen and phosphorus, is generally the major limitation to plant growth. A number of plant species have evolved beneficial interactions with micro-organisms that facilitate the uptake of these nutrients. Legumes form symbiotic interactions with mycorrhizal fungi that facilitate phosphate uptake and with rhizobial bacteria that provide the plant with a source of nitrogen. The establishment of these symbioses involves a molecular communication between the plant and the symbiotic micro-organisms in the soil. Mycorrhizal fungi and rhizobial bacteria release signals that are recognised by the host plant and lead to developmental changes associated with the accommodation of the symbionts. Genetic dissection in legumes has defined the signalling pathways involved in these symbioses and this signalling process involves oscillations in calcium within the nuclear region. Our work has been focused on understanding how plant perception of symbiotic signalling molecules leads to the activation of nuclear calcium oscillations and how the perception of these calcium oscillations drives the developmental changes associated with symbiosis. Using this knowledge on the symbiotic systems of legumes we are attempting to engineer these signalling processes into cereal crops as the first step in engineering nitrogen-fixing cereals.

The Broader Benefits of N_2-fixation

Ken E. Giller

Plant Production Systems, Wageningen University, The Netherlands

Symbiotic N_2-fixation in legumes contributes to the global economy and human well being in many ways. Apart from the direct benefits of N_2-fixation to food production and human nutrition, the N_2-fixation by legumes plays a major role in provision of feed concentrates and fodder for animals, in maintenance of soil fertility and a further role in provision of other products: valuable timber, stakes and secondary metabolites. In this talk I will review these broader benefits, with particular emphasis on how N_2-fixation contributes to smallholder agriculture in the tropics.



POSTER SESSION 1

Signal Perception and Transduction

Room Orion

POSTER 1-1 /LIGHTNING TALK/

From the Genetic Map to the Genome Assembly of the Nod Factor-independent *Aeschynomene evenia* to Shed Light on the Evolution of Nodulation

P. Leleux[1,2], C. Chaintreuil[1], L. Lamy[1,2], R. Rivallan[3], C. Klopp[2], D. J. Bertioli[4], E. Giraud[1], Jean-Francois Arrighi[1]

[1] *IRD, Laboratoire des Symbioses Tropicales et Méditerranéennes, Campus International de Baillarguet, Montpellier, France*
[2] *INRA, Plateforme GenoToul Bioinfo, INRA Auzeville, Castanet-Tolosan, France*
[3] *CIRAD, UMR AGAP, Campus de Lavalette, Montpellier, France*
[4] *University of Brasilia, Institute of Biological Sciences, Campus Darcy Ribeiro, Brasilia, Brazil*

Aeschynomene evenia has emerged as a new model legume for the deciphering of the molecular mechanisms of an alternative symbiotic process that is independent of Nod factors (1-2). Whereas most of the research on nitrogen-fixing symbiosis, legume genetics and genomics has so far focused on Galegoid and Phaseolid legumes, *A. evenia* falls in the more basal and understudied Dalbergioid clade along with peanut (*Arachis hypogaea*). In a first step to provide insights into the symbiotic genes content and the structure of the *A. evenia* genome (2n=20, 415 Mb), a genetic map was developed (3). For this, an RNAseq analysis was performed, allowing the development of molecular markers and the identification of most, but not all, symbiotic genes. Altogether, they were used to genotype a F2 mapping population, resulting in a gene-based genetic map (364 markers, 1036 cM) that was arranged in 10 linkage groups. Comparative genomic analysis with the sequenced *Arachis* genomes also indicated they are constituted of blocks of conserved macrosynteny. Thus, this genetic-map revealed the structure of the genome gene-space and, in particular, uncovered the distribution of expressed orthologs of known symbiotic genes. To further advance in our understanding of the evolution of nodulation and legume genomes, we are now engaged in an *Aeschynomene* genome sequencing project. Using the PacBio technology, a ~78x sequencing coverage of the *A. evenia* genome was obtained, resulting in a scaffold assembly and anchoring to the genetic map that represented 92% and 81% of the *A. evenia* genome size, respectively. The forthcoming completion of a reference genome sequence for *A. evenia* is anticipated to shed light on the evolution of symbiotic genes that could not be found in the *A. evenia* transcriptome datasets. It should also fasten the identification of molecular determinants of the Nod factor-independent process thanks to an ongoing mutagenesis approach by coupling gene mapping with sequencing at gene or whole genome-level.

References:

[1] *Arrighi et al. (2012) MPMI. 25: 851-861.*
[2] *Arrighi et al. (2013) Plos One. 8: e63836.*
[3] *Chaintreuil et al. (submitted).*

POSTER 1-2 /LIGHTNING TALK/

The Pea (*Pisum sativum* L.) Receptor-like Kinase Gene *LykX*, the Most Prominent Candidate for *Sym2*, is Required for Successful Penetration of Rhizobia into the Root Hair

Anton Sulima[1], Vladimir Zhukov[1], Alexey Borisov[1], Igor Tikhonovich[1,2]

[1] *All-Russia Research Institute for Agricultural Microbiology, Saint-Petersburg, Russian Federation*
[2] *Saint-Petersburg State University, Department of Genetics and Biotechnology, Saint-Petersburg, Russian Federation*

Specificity of the symbiosis between legume plants (Fabaceae) and nodule bacteria (polyphyletic group collectively known as rhizobia) is based on ligand-receptor interactions, during which the bacterial signal molecules (Nod factors) are recognized by LysM-containing plant receptor kinases (1,2). Within the pea (*Pisum sativum* L.) species, the group of genotypes originating from Middle East (so-called "Afghan peas") exists with increased selectivity towards Nod factor structure (3). This trait is controlled by plant gene *Sym2* localized in I linkage group (LG I) of pea genome and presumably encoding the Nod factor receptor (4).

Screening of pea genome BAC library (in collaboration with Dr. Helene Berges, CNRGV, France) revealed new pea gene *LykX* which is located in the *Sym2* region and encodes a receptor-like LysM kinase. We have found two specific allelic states of *LykX* resulting into varieties in amino acid composition of LysM motiff which perfectly correlate with the high or low selectivity in legume-rhizobial symbiosis. Thus, *LykX* is currently considered the most likely candidate for the *Sym2*.

For a further description of the role of *LykX* in symbiosis we ordered the TILLING of pea mutant collection (in collaboration with Dr. Marion Dalmais, INRA-URGV, France). 8 mutant families with missence mutations presumably disrupting the function of LykX protein (according to the *in silico* prediction; SIFT program) were identified. After the inoculation with *Rhizobium leguminosarum* bv. *viciae* strain RCAM1026 plants in each family have shown the decreased number of nodules along with significantly increased number of infection attempts (cases of bacterial penetration into the infection thread) in comparison to wild type plants. The earlier stages of the symbiosis appeared to be unaffected, and the nodules formed were phenotypically identical to those of wild type. To decisively confirm the role of *LykX* in selectivity towards Nod factor structure, we intend to conduct the allelism test between *lykX* mutants and "Afghan" forms of pea.

This work was supported by RSF grant 14-24-00135, RFBR grant 15-29-02737 and Grant of President NSH6759.2016.4.

References:

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[3] *Razumovskaya Z.G. (1937) Russian J Microbiol. 6:321-328.*
[4] *Kozik et al. (1996) Plant Mol Biol. 31(1):149-156.*

POSTER 1-3 /LIGHTNING TALK/

Lotus-Rhizobium Symbiosis is Facilitated by the Epidermal Nod Factor Receptor

Ei-ichi Murakami, Jeryl Cheng, Zoltán Bozsóki, Kira Gysel, Yasuyuki Kawaharada, Lene H. Madsen, Jens Stougaard and Simona Radutoiu

Department of Molecular Biology and Genetics, Aarhus University, Denmark

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Nitrogen fixing symbiosis initiates in most legumes in the epidermis where a more relaxed sensitivity for the structure of bacterial Nod factors exists, compared to the later stages of the interaction which unfold in the inner cortical layers (1). Identifying components that contribute to this differential sensitivity is essential for understanding how legume-rhizobia specificity is acquired. In *Lotus japonicus*, nanomolar concentrations of *M.loti* Nod factors are detected in the root hairs by the NFR1 and NFR5 receptor kinases that control the tightly coordinated molecular, cellular and physiological events leading to root nodule symbiosis (2, 3). NFR1 and NFR5 are the founders of LysM receptor kinase family, that in legumes has greatly expanded through whole genome or tandem duplications (4). The central role of NFR1 and NFR5 in root nodule symbiosis concealed so far, the contribution of the other LysM receptors to the symbiotic development. We have identified an additional LysM receptor that contributes to Nod factor perception and signalling in the epidermis. Our results obtained from phenotypic analyses of mutant plants coupled with tailored genetic complementation studies, and biochemical investigations indicate that complex signalling hubs are assembled at different stages of the symbiosis, where the assortment and contribution of LysM receptors is regulated transcriptionally and biochemically.

References:

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[2] *Radutoiu S., et al. Nature, 2003.*
[3] *Broghammer A., et al., PNAS. 2012*
[4] *Lohmann GV. et al., Mol Plant Microbe Interact. 2010.*

POSTER 1-4 /LIGHTNING TALK/

Identification of a Novel Component of the CCaMK/CYCLOPS Complex

Katja Katzer and Martin Parniske

University of Munich (LMU), Faculty of Biology, Genetics, Martinsried, Germany

Early developmental stages of the agriculturally and ecologically important plant root symbioses with phosphate-acquiring arbuscular mycorrhiza (AM) fungi and nitrogen-fixing bacteria share common signaling components. A complex of a calcium- and calmodulin-dependent kinase (CCaMK) and the transcriptional regulator CYCLOPS represents the last step of the common symbiotic pathway before the bifurcation of signal transduction, but the mechanism underlying the developmental decision process remained unknown. Several lines of evidence indicate that additional complex components are involved in tuning the activity of the complex. Here we provide the first structural insights into CYCLOPS regulation during root symbiosis. Using x-ray crystallography, part of the CYCLOPS DNA-binding domain was structurally solved. Based on the protein structure, a novel component of the CCaMK/CYCLOPS complex could be predicted and interactions confirmed by independent protein-protein and protein-DNA interaction assays. Mutational analyses to weaken or strengthen the conformational integrity, demonstrated the importance of the structure for CYCLOPS' activity and regulation.

POSTER 1-5 /LIGHTNING TALK/

KNAT3/4/5-like KNOX Transcription Factors Regulate Symbiotic Nodule Organ Development in *Medicago truncatula* Potentially Through the *MtEFD*/*MtRR4* Cytokinin-Related Regulatory Module

Carole Laffont[1], Elisabetta Di Giacomo[2], Francesca Sciarra[2], Maria Adelaide Iannelli[2], Florian Frugier[1] and Giovanna Frugis[1]

[1] *Institute of Plant Sciences Paris-Saclay (IPS2), CNRS, INRA, Université Paris Sud, Université Evry, Université Paris-Diderot, Université Paris-Saclay, Gif-sur-Yvette, France*
[2] *Istituto di Biologia e Biotecnologia Agraria (IBBA), Operative Unit of Rome, Consiglio Nazionale delle Ricerche (CNR), Monterotondo Scalo (Roma), Italy*

Class 1 KNOX homeodomain transcription factors (TFs) are involved in plant shoot development and leaf shape diversity, whereas Class 2 *KNOX* genes are less characterized, even though an antagonistic function relative to Class 1 KNOXs was recently proposed. We investigated the role of *KNOX* genes in legume root nodule organogenesis using the *Medicago truncatula* model. *In silico* expression data together with GUS transcriptional fusions identified three *Mt KNAT3/4/5-like* genes expressed during nodulation from the early primordia stages. *Mt KNAT3/4/5-like* genes encode four highly homologous proteins expressed during nodule organogenesis and in overlapping zones of the nodule, suggesting functional redundancy. A simultaneous RNAi-mediated silencing of *Mt KNAT3/4/5-like* genes provoked an increased formation of fused nodule organs, correlated with a decreased expression of two genes associated to nodule organogenesis: the *MtEFD* (*Ethylene response Factor required for nodule Differentiation*) TF (1) and its direct target *MtRR4,* a type A cytokinin Response Regulator gene expressed in nodule primordia (2). This suggests that *Mt KNAT3/4/5-like* genes therefore regulate legume nodule development potentially through the MtEFD/MtRR4 cytokinin-related regulatory module and may contribute to the diversity of nodule shapes.

References:

[1] *Vernié et al., 2008, Plant Cell, 20:2696-271*
[2] *(Plet et al., 2011, Plant J., 65:622-633).*

POSTER 1-6 /LIGHTNING TALK/

Nodule and Lateral Root Development Are Mediated by Independent Pathways Downstream of the MtCEP1 Peptide / CRA2 Receptor in *Medicago truncatula*

Nadiatul A. Mohd Radzman[1], Carole Laffont[2], Ariel Ivanovici[1], Neha Patel[1], Dugald Reid[3], Jens Stougaard[3], Florian Frugier[2], Nijat Imin[1] and Michael A. Djordjevic[1]

[1] *Plant Science Division, Research School of Biology, College of Medicine, Biology and the Environment, Australian National University, Canberra, Australia*
[2] *Institute of Plant Sciences Paris-Saclay (IPS2), CNRS, INRA, Univ Paris Sud, Univ Evry, Univ Paris-Diderot, Université Paris-Saclay, Gif sur Yvette, France*
[3] *Plant Molecular Biology, Department of Molecular Biology and Genetics, Aarhus, Denmark*

C-TERMINALLY ENCODED PEPTIDEs (CEPs) are important non-cell-autonomous regulators of root system architecture that are secreted to the apoplast (1-3). In *Medicago truncatula* (*Mt*), MtCEP1 is upregulated in roots by nitrogen limitation which is required for nodulation competency. *MtCEP1* over expression or addition of MtCEP1 peptide to roots increases nodule number and inhibits lateral root emergence (1, 2). MtCEP1 peptide-dependent nodulation phenotypes were found to require the symbiotic (SYM) signalling pathway and MtEIN2 (Mt ETHYLENE INSENSITIVE 2) but acted independently of SUNN. MtCEP1 inhibition of lateral root emergence acted through a separate and MtEIN2-independent mechanism. We investigated how MtCEP1 enhances nodulation. Raising MtCEP1 levels increases the number of rhizobial infections, extends the developmental competency of roots for nodulation and leads to the formation of "fused" nodules with multiple nodule meristems. Nodule formation occurs at both proto-xylem and proto-phloem poles. These results suggest that the MtCEP1 peptide decreases MtEIN2-dependent responses that negatively regulate nodule formation. Accordingly, MtCEP1 counteracts the phenotypic effects of increasing ethylene precursor concentrations and an ethylene synthesis inhibitor treatment antagonises MtCEP1 nodulation phenotypes. MtCEP1 also inhibits the development of MtEIN2-dependent pseudonodule formation. A mutant affecting the MtCRA2 (COMPACT ROOT ARCHITECTURE 2) receptor was examined due to its close homology to the *Arabidopsis* CEP Receptor 1 (4). The *cra2* mutant was found to be unresponsive to MtCEP1 effects on lateral root and nodule formation. This suggests CRA2 is a CEP peptide receptor that mediates both organogenesis programs. In addition, an ethylene inhibitor treatment complements the *cra2* nodulation, but not the lateral root phenotypes. These results indicate that MtCEP1 and its likely receptor, CRA2, act through ethylene-dependent and independent pathways to regulate important aspects of root system architecture.

References:

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POSTER 1-7

Characterisation of the Novel *Lotus japonicus* Symbiotic Mutant EXO422

Huijun Liu, Yasuyuki Kawaharada, Simon Kelly, Niels Sandal, Stig U. Andersen and Jens Stougaard

The Centre for Carbohydrate Recognition and Signalling
Department of Molecular Biology and Genetics, Aarhus University

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The symbiotic interaction between legume plants and soil bacteria, collectively referred to as rhizobia, results in the formation of root nodules within which rhizobia convert atmospheric nitrogen into forms biologically available to the plant. In return, the rhizobia are provided with carbon sources. Although well studied, the mechanisms and signalling pathways that allow the plant to co-ordinate rhizobia infection and nodule organogenesis remain incomplete.

To further investigate these mechanisms, a symbiotic mutant isolated from a random mutagenesis screen of *Lotus japonicus* Gifu is being characterised. The EXO422 mutant forms large–uninfected nodules when inoculated with the expolysaccharide mutant *Mesorhizobium loti* R7A *exoU*. In contrast, Gifu plants form only small-uninfected nodule primordia. Infection thread formation by *exoU* is similarly impaired on both Gifu and EXO422 mutant plants. Wild-type *M. loti* R7A, forms less infection threads and shows reduced nodulation rates on EXO422 compared to Gifu. The symbiotic phenotypes indicate that EXO422 is impaired in the signalling that co-ordinates the infection process and nodule development. EXO422 shows additional phenotypes including the formation of short roots that harbor many root hairs. These phenotypes are likely related to observed differences in ethylene production. Furthermore, EXO422 forms spontaneous nodules in the absence of rhizobia. Rough mapping of EXO422 indicates that the mutation responsible for the large–uninfected nodule phenotype in association with *exoU* is located at the top of chromosome 2. Mapping of the spontaneous nodule phenotype indicates the responsible mutation is at the end of chromosome 4. These mapping results indicate that the phenotypes exhibited by EXO422 are due to multiple mutations.

POSTER 1-8

Characterizing the Role of Cytokinin Transport in *Lotus japonicus* Nodule Development

Yumeng Chen, Flavien Buron, Marcin Nadzieja, Jens Stougaard, Dugald Reid

Centre for Carbohydrate Recognition and Signalling, Department of Molecular Biology and Genetics, Aarhus University, Denmark

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The identification of several classes of cytokinin transporters indicates that short and long distance transport are likely required for regulating cytokinin localization and signaling. Legume nodule development provides a good system to study cytokinin transport, as cytokinin is both necessary and sufficient for the cortical cell divisions observed during this process. This study will characterize the role of transporters of the Purine Permease (PUP) family during symbiotic nodule development in *Lotus japonicus*. Transcript profiling and *pPUP-GUS* reporter gene studies showed several *LjPUPs* are expressed during nodule development, especially *PUP1* and *PUP3*, in nodules. We have isolated *PUP1*, *PUP2*, *PUP3* and *PUP4* single mutants with Lotus Endogenous Retrotransposon1 (LORE1) insertions, which are being used for constructing multiple mutants and further phenotypic and functional analyses. Lines overexpressing PUP will be created (*LjUbi-PUP*) for additional analyses. We will also identify the subcellular localization of LjPUPs through fusion proteins composed of LjPUP-GFP with the aim of performing transport assays in tobacco microsomes. To explore the role of LjPUPs in cytokinin homeostasis and signalling, *LjPUPs* and cytokinin signalling genes both in *pup* and wide type will be assayed using quantitative RT-PCR. Together, it is expected the functional analysis of genes of the PUP family will help to clarify the role and effects of cytokinin transporters during nodule development in *L. japonicus* and may have implications for the understanding of regulation of cytokinin homeostasis in other plants.

POSTER 1-9

DII-based Auxin Accumulation Sensor Reveals a Novel Auxin Contribution to the Symbiotic Infection in *Lotus japonicus*

Marcin Nadzieja, Simon Kelly, Jens Stougaard, Dugald Reid

Aarhus University, Department of Molecular Biology and Genetics, Centre for Carbohydrate Recognition and Signalling, Aarhus C, Denmark

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The plant hormone auxin contributes to the phenomenon of root nodulation. In *Lotus japonicus*, auxin maxima are observed in nodule primordia and accompany cell divisions in root cortex (1). Application of auxin transport inhibitors is also sufficient to trigger nodule formation is some legumes (2). More recently, a requirement for auxin signalling during symbiotic infection was shown (3). However, the precise timing of auxin accumulation during nodulation, auxin interplay with other plant hormones and the genetic network governing auxin responses remain unclear.

We developed a robust *L. japonicus* variant of the recently described DII-based sensor for auxin accumulation (4). We used the sensor together with *DR5* auxin responsive promoter to follow auxin dynamics in *L. japonicus* roots with high spatiotemporal resolution. We found that auxin accumulates in root hairs upon infection. Moreover, nod factor alone is sufficient for producing this response. We identified two genes in auxin biosynthesis pathway to be upregulated shortly after nod factor treatment. We were also able to demonstrate through chemical inhibition of auxin biosynthesis a functional role for biosynthesis of auxin in infection events. Additionally, we observed auxin accumulation in deep cell layers, namely pericycle and endodermis, situated on infected sides of root. With use of gravitropism, we mimicked this pattern of auxin accumulation and observed clear effect on positioning of nodules. Thus, we showed that alteration of auxin homeostasis by, presumably, regulation of auxin transport, influences nodule organogenesis.

In this project we developed and characterized tools in order to provide greater detail of hormone dynamics, in particular auxin, during infection and organogenesis.

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POSTER 1-10

Discovery of Interaction Partners of Nod Factor Receptor 5 (NFR5) in *Lotus japonicus*

Jaslyn Wong[1], Mette Berentsen[1], Daniel Couto[2], Paul Derbyshire[2], Christoph Bücherl[2], Mette Asmussen[1], Niels Sandal[1], Frank Menke[2], Søren Thirup[1], Simona Radutoiu[1], Mickaël Blaise[1], Cyril Zipfel[2], Jens Stougaard[1]

[1] *Centre for Carbohydrate Recognition and Signalling, Department of Molecular Biology and Genetics, Aarhus University, Gustav Wieds Vej 10C, 8000 Aarhus C, Denmark*
[2] *The Sainsbury Laboratory, Colney Lane, Norwich NR4 7UH, United Kingdom*

During rhizobium-legume symbiosis, rhizobia secrete specific lipo-chitooligosaccharides termed nodulation (nod) factors. Perception of these nod factors by Nod Factor Receptor 1 and 5 (NFR1 and NFR5) triggers the downstream signalling in *Lotus japonicus*. Genetic studies have shown that the plasma membrane-localized Symbiosis Receptor Kinase (SymRK), as well as nuclear-localized cation channels and nucleoporins are required for calcium oscillations in the perinuclear space of root hairs exposed to nod factor. The calcium signal is subsequently decoded by the nuclear Calcium/Calmodulin-dependent Kinase (CCaMK), which then phosphorylates transcriptional activators and consequently activates transcriptional changes leading to nodule and infection thread formation.

Taking a novel approach we have attempted to identify additional components linking nod factor perception at the plasma membrane to calcium oscillations and transcriptional changes in the nucleus. To identify such components, co-immunoprecipitation experiments were performed using *Lotus* lines that stably express NFR5-eYFP fusion proteins. Analysing the co-immunoprecipitated complexes on an Orbitrap Fusion Tribrid mass spectrophotometer led to the discovery of novel nod factor-dependent and/or independent NFR5-eYFP-interacting proteins. Current efforts are focused on validating these interactions, and on characterising the roles of these interactors in symbiosis signaling.

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POSTER 1-11

Engineering Nodulation Signalling in Barley

Doreen Feike[1], Ben Miller[2], Andrey Korolev[1], Eleni Soumpourou[1], Andy Breakspear[1], Tom Lawrenson[1], Wendy Harwood[1], Christian Rogers[1], Giles Oldroyd[1]

[1] *John Innes Centre Norwich, Norwich Research Park, Colney Lane, United Kingdom*
[2] *University of East Anglia, Norwich Research Park, Norwich, Norfolk, United Kingdom*

Legumes form symbiotic associations with rhizobia and mycorrhizal fungi. Both symbionts produce signalling molecules that are detected by specific plant plasma-membrane receptors. These receptors activate the common symbiosis signalling pathway (SYM). A characteristic hallmark of SYM activation is the occurrence of nuclear calcium spiking that occurs within minutes of signal application. A calcium and calmodulin-dependent protein kinase (CCaMK) and a transcription factor CYCLOPS are thought to decode this signal to activate either nodulation- or mycorrhization- specific gene expression. The SYM pathway is also present in cereals and essential for mycorrhizal signalling. Adapting it for the recognition of rhizobia and the initiation of nodulation might reduce the need for nitrogenous fertilizer, improve yields and benefit the environment. This is the goal of the ENSA (Engineering Nitrogen Symbiosis for Africa) project.

One objective of ENSA is the engineering of CCaMK and the transcription factors CYCLOPS, ERN1, NIN, NSP1, NSP2 into the model cereal plant barley. All of these transcription factors are required for nodulation in legumes. Our short-term goal is to express them in barley and to verify their activity *in vivo* using promoter-reporter fusions. We then want to investigate the effects of their expression on (symbiotic) gene expression in barley. Further, we aim to build transcription factor cascades to mimic the activation of the legume nodulation-signalling pathway. During the last 3 years, we have focussed on generating the tools for gene stacking in barley. We established Golden Gate cloning for the assembly of hundreds of multi-gene constructs, which have been successfully transformed into barley. We have gathered a library of promoters and terminators which we tested for their suitability for barley engineering. We also investigated the effects of codon-optimisation and intron-mediated enhancement on protein levels. We have created a large number of stable barley transgenic lines and we will report on the impact of nodulation transcription factor engineering in barley.

POSTER 1-12

Functional Diversification of Duplicated *EIN2* in *Lotus japonicus*

Terry Mun, Sara Moeskjær, Jens Stougaard, and Stig U. Andersen

Centre for Carbohydrate Recognition and Signalling, Aarhus University, Denmark

Lotus japonicus is able to establish a well-characterized nitrogen-fixing symbiosis upon inoculation with a compatible symbiont, *Mesorhizobium loti* (1). It has been previously discovered that *L. japonicus* have two paralogous copies of the *Ein2* gene, which is involved in nuclear signal transduction in the presence of ethylene (3).

As ethylene is a pleotropic phytohormone, we generated homozygous insertional mutants of both copies of *LjEin2* from the *LORE1* population (4,5) and scored root growth, lateral root formation and nodulation rate at 3 weeks post-inoculation (wpi). Both *ein2a* and *ein2b* mutants show inhibitions in root growth and lateral root formation upon ACC treatment, suggesting that these two mutants remain ACC sensitive. In addition, ACC treatment also led to reduction in nodulation rates in both mutants. However, *ein2b* showed significantly higher rates of nodulation compared to wild type Gifu and *ein2a* mutants, suggesting that *ein2b*, but not *ein2a*, restricts nodule formation. Moreover, the observation that *ein2b* remains ACC sensitive suggests that both *LjEin2* copies share a common upstream signal transduction pathway, but have different downstream targets.

In order to further clarify the origins of *Ein2* duplication, we constructed a phylogenic tree using *Ein2* homologs found across various model plants. Our analysis shows that leguminous species that form indeterminate nodules harbors only a single copy of *Ein2*, while those that form determinate nodules have multiple paralogs of the gene. More interestingly, among determinate nodulating legumes, inter-species orthologous copies of *Ein2* are more similar to each other than to their respective paralogs. This observation suggests that (a) either legumes carrying duplicated *Ein2*-like genes have lost all but one copy through convergent evolution, (b) or that a common gene duplication event has occurred in the ancestor of such legumes as a form of divergent evolution.

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POSTER 1-13

Gatekeepers of Rhizobia Entry: Cytokinin-ethylene Crosstalk Regulates Infection in *Lotus japonicus*

Mandana Miri[1,2] and Krzysztof Szczyglowski[1,2]

[1] *Agriculture and Agri-Food Canada, SCPFRC, London, Ontario, Canada*
[2] *Department of Biology, University of Western Ontario, London, Ontario, Canada*

Leguminous plants selectively initiate primary responses to rhizobial nodulation factors which ultimately lead to symbiotic root nodule formation. The signaling pathways for nodule development control two major events, rhizobial infection (epidermal program) and nodule organogenesis (cortical program). The role of cytokinin as the key endogenous plant inducer of nodule differentiation has now been firmly established [1, 2]. In the model legume *Lotus japonicus,* this process is mediated by the Lotus Histidine Kinase (LHK1) cytokinin receptor with a partially redundant involvement of LHK1a and LHK3 [3]. However, the role of cytokinin in mediating rhizobial entry into roots is less clear. Our data indicate that in *L. japonicus*, cytokinin receptors have no apparent function during the initial epidermal infection thread formation events. However, in their absence, in the *lhk1-1 lhk1a-1 lhk3-1* triple receptor mutant, cortical infection threads do not develop [3, 4]. Thus, cytokinin might be the primary, plant endogenous signal that conditions cortical cells for upcoming rhizobial infections [4]. Interestingly, the cytokinin-dependent signaling events which promote the development of infection threads in the cortex also appear to stimulate a negative feedback mechanism that restrict subsequent infections at the root epidermis [4] which presumably is the result of cytokinin-ethylene crosstalk. As the rate-limiting step in ethylene biosynthesis is mediated by ACC synthase (ACS), we hypothesize that increased cytokinin activity in the root epidermis, as mediated by LHK1, enhances the ACS activity. This is presumed to elevate the ethylene level, which subsequently inhibits bacterial infection. We have identified a family of at least seven *ACS* genes in *L. japonicus.* By conducting various types of expression analyses we have shown that *ACS1* and *ACS2* are potential targets of LHK1-dependent signaling. Furthermore, using a combined approach involving reverse genetic and expression analyses, the role of *ACS1* and *ACS2* in the *lhk1-1* hyperinfection phenotype has been further investigated, which may clarify how cytokinin-ethylene crosstalk regulates the extent of bacterial infection in legume plants. A model for cytokinin-dependent regulation of *Mesohizobium loti* infection in *L. japonicus* will be presented.

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POSTER 1-14

Identifying Downstream Effects of *Lotus japonicus* Exopolysaccharide Receptor EPR3

Simon Kelly, Yasuyuki Kawaharada, Vikas Gupta, Niraj Shah, Stig Andersen, Simona Radutoiu, Jens Stougaard

Centre for Carbohydrate Recognition and Signalling, Department of Molecular Biology and Genetics, Aarhus University, Denmark

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The symbiotic interaction between legumes and rhizobia requires complex molecular communication between the two partners to determine compatibility and ensure a successful outcome, the formation of nitrogen-fixing root nodules. In addition to the major signal molecules produced by rhizobia, lipo-chitooligosaccharides (Nod factors), further signalling plays a role in progression of the symbiotic process. Recently, a *Lotus japonicus* receptor (EPR3) for *Mesorhizobium loti* exopolysaccharide (EPS) was identified[1].

In order to determine which *Lotus* components are affected downstream of EPR3/EPS interactions several approaches have been pursued.

RNA-seq based transcriptome approaches have been employed to identify gene regulation changes in root hairs at early-stages (24 & 72 hpi) and from young nodule primordia at later-stages (7 dpi) of symbiotic interactions. Wild-type and *epr3* mutant plants were examined in these studies following inoculation with wild-type *M. loti* R7A or EPS-deficient strains R7AexoB and R7AexoU[1,2]. Candidate genes that were identified from the RNA-seq data are being confirmed by qPCR analysis and followed up through observations of promoter-reporter gene fusions in transgenic roots at various stages of symbiotic interactions. Additionally, LORE1 retrotransposon mutants of candidate genes are being propagated for phenotypic characterisation.

In an alternative approach, the phosphoproteome of *Lotus* is being examined. Wild-type R7A and R7AexoU, which is severely impaired in the nodulation of wild-type *Lotus* but is able to nodulate *epr3* mutants, are initially being investigated.

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POSTER 1-15

KNOX3 as a Possible Activator of Cytokinin Biosynthesis Genes in *Medicago truncatula*

Mahboobeh Azarakhsh, Maria A. Lebedeva, Ludmila A. Lutova

Department of Genetics and Biotechnology, St Petersburg State University, St Petersburg, Russia

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KNOX transcription factors regulate different aspects of plant development essentially through their effects on phytohormones. We found that KNOX transcription factor KNOX3 is involved in nodule development in Medicago truncatula. KNOX3 ectopic expression caused the formation of nodule-like structures in the absence of rhizobia, a phenotype previously observed in mutants with a constant activation of cytokinin receptor. We hypothesized that KNOX3 may be responsible for cytokinin pathway activation upon nodulation.

Our data on down regulation and overexpression of KNOX3 suggest that in nodules KNOX3 might be responsible for activation of cytokinin biosynthesis genes, i.e. isopen*tenyl transferase* (*IPTs*) and *LONELY GUY* (*LOG*) during nodulation.

To check KNOX3 direct binding with its possible targets in Medicago truncatula a set of approaches such as EMSA (electrophoretic mobility shift assay), SPR (surface plasmon resonance) and ChIP will be used.

So, similarly to shoot apical meristem, where KNOX transcription factors are responsible for activation of cytokinin biosynthesis genes, KNOX3 might have an analogous role during symbiotic nodule development.

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POSTER 1-16

MicroRNA167-directed Regulation of the Auxin Response Factors GmARF8a and GmARF8b is Required for Soybean Nodulation and Lateral Root Development

You N. Wang[1], KX Li[1], BJ Ferguson[2], PM Gresshoff[2] and Xia Li[1]

[1] *College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, China*
[2] *Centre for Integrative Legume Research, University of Queensland, Queensland, Australia*
xli@mail.hzau.edu.cn

Legume root nodules convert atmospheric nitrogen gas into ammonium through symbiosis with a prokaryotic microsymbiont broadly called rhizobia. Auxin signaling is required for determinant nodule development; however, the molecular mechanism of auxin-mediated nodule formation remains largely unknown.

Here, we show in soybean (*Glycine max*) that the microRNA miR167 acts as a positive regulator of lateral root organs, namely nodules and lateral roots. miR167c expression was upregulated in the vasculature, pericycle, and cortex of soybean roots following inoculation with *Bradyrhizobium japonicum* strain USDA110. It was found to positively regulate nodule numbers directly by repressing the target genes GmARF8a and GmARF8b. Moreover, the expression of miR167 and its targets was up- and down-regulated by auxin, respectively. The miR167-GmARF8 module also positively regulated nodulation efficiency under low microsymbiont density, a condition often associated with environmental stress. The regulatory role of miR167 on nodule initiation was dependent on the Nod factor receptor GmNFR1α, and it acts upstream of the nodulation-associated genes, such as *NIN* and *ENOD40-1*. miR167 also promoted lateral root numbers.

Collectively, our findings establish a key role for the miR167-GmARF8 module in auxin mediated nodule and lateral root formation in soybean.

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POSTER 1-17

Nodulation Suppressive Glycosylated CLE Peptides in *Glycine Max* and *Pisum sativum*

April H. Hastwell[1], Leo Corcilius[2], Mengbai Zhang[1], Candice Jones[1], Thomas de Bang[3,4], Richard J. Payne[2], Peter M Gresshoff[1], Brett J Ferguson[1]

[1] *Centre for Integrative Legume Research, School of Agriculture and Food Sciences, The University of Queensland, St Lucia, Brisbane, Australia*
[2] *School of Chemistry, The University of Sydney, Sydney, Australia*
[3] *Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, United States*
[4] *current address: Section for Plant and Soil Sciences, Faculty of Science, University of Copenhagen, Frederiksberg C, Denmark*

Members of the CLAVATA3/EMBRYO SURROUNDING REGION-related (CLE) peptide family play important roles in maintaining meristematic homeostasis in a range of developmental stages and tissue types. We recently identified the complete CLE peptide families of four legume species: soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), *Medicago truncatula* and *Lotus japonicus* (1). This is particularly important, as several CLE peptides are key molecular signals in controlling rhizobia colonisation and preventing the bacteria from excessively draining resources from the host plant (2-4). Autoregulation of Nodulation (AON) is the systemic mechanism used by legumes to inhibit excessive nodule development. The molecular pathway begins with the production of a CLE peptide in response to the first cell divisions following infection by compatible rhizobia (2). The CLE peptide is cleaved from the prepropeptide, hydroxylated, glycosylated and transported to the shoot where it interacts with the LRR receptor kinase called Nodulation Autoregulation Receptor Kinase (NARK), SUNN and HAR1 (2-4). The AON mechanism is highly conserved across legume species; this was elegantly demonstrated by overexpressing soybean *Rhizobia-Induced CLE 1a* (*GmRIC1a*) in common bean. GmRIC1a was able to completely suppress nodulation interspecifically in WT, but not in the supernodulation *NARK* mutant, R32 (5).

Other CLE peptide-encoding genes are expressed in response to soil nutrients, such as nitrate (3,4). In plants overexpressing *Nitrate-Induced CLE1a* (*GmNIC1a*), nodulation is not completely suppressed as with *GmRIC1a* (3). The mechanism for incomplete nodule suppression by *GmNIC1a* relates to its inability to travel systemically within the plant. Post-translational modifications, such as glycosylation, of the CLE peptides are also very important to their function (4). Peptides fed to legume plants devoid of proper modifications are unable to suppress nodulation. The effect of glycosylated peptides on nodulation was also examined.

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POSTER 1-18

Production of Nod Factors by the Gamma-proteobacterium *Pseudomonas protegens*

Kevin Garcia[1], Min-Hyung Ryu[2], Junko Maeda[1], Matthew B. Crook[1], Christopher Voigt[2], Jean-Michel Ané[1]

[1] *Departments of Bacteriology and Agronomy, University of Wisconsin-Madison, Madison, United States*

[2] *Synthetic Biology Center, Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, United States*

Nodulation factors are lipochitooligosaccharides (LCOs) required by most rhizobia for the colonization of legume roots and nodule development. These LCOs are active at very low concentrations and substitutions on these LCOs are the main determinant of host specificity in the rhizobia – legume symbiosis. In addition, LCOs have been shown to promote root development and are commercialized as plant growth stimulants. LCOs are naturally produced by alpha- and beta-proteobacteria and encoded by conserved *nod* genes. *Rhizobium* sp. IRBG74 produces non-sulfated LCOs with arabinose, fucose and methyl-fucose substitutions. It is able to nodulate diverse legume hosts including at least eight different *Sesbania* species as well as the model legume, *Lotus japonicus*. In contrast, *Pseudomonas protegens* Pf-5 is a gamma-proteobacterium that promotes growth on a wide range of plant species but does not form nodules on legume roots. In order to construct a modular cluster that can be transferred to other beneficial bacteria and produce LCOs only when required by the plants, we built a synthetic LCOs-producing cluster composed of multiple artificial operons made up of 21 genes from *Rhizobium* sp. IRBG74, synthetic terminators and T7 promoters. Inoculation of several engineered *P. protegens* Pf-5 strains on *Lotus japonicus* and common vetch (*Vicia sativa*) roots led to extensive root hair deformations (branching) similar to those observed with purified LCOs from *Rhizobium* sp. IRBG74. As controls, *P. protegens* Pf-5 without any refactored LCO cluster or purified chitin oligomers did not trigger any root hair deformations indicating the production of LCOs by the engineered *P. protegens* Pf-5. Progress towards the characterization of LCOs produced, plant growth promoting effects, and the colonization of legumes by these *P. protegens* Pf-5 strains will be presented.

POSTER 1-19

Regulation of Small RNAs and Corresponding Targets in Nod Factors-induced *Phaseolus Vulgaris* Root Hair Cells

Damien Formey[1], Jose A. Martín-Rodríguez[1], Alfonso Leija[1], Olivia Santana[2], Luis Cárdenas[2] and Georgina Hernández[1]

[1] *Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México (UNAM), Cuernavaca, Morelos, México*
[2] *Instituto de Biotecnología, Universidad Nacional Autónoma de México (UNAM), Cuernavaca, Morelos, México*

A *Phasolus vulgaris* genome-wide analysis led to the identification of the catalog of small RNAS (sRNA) of this agronomical important legume. This revealed newly identified *P.* vulgaris-specific microRNAs (miRNAs) that could be involved in the regulation of the rhizobia-symbiotic process (1). Our current work focuses on the functional analysis of these molecules. Generally, novel miRNAs are difficult to identify and study because they are expressed very low and in a tissue- or cell-specific manner. To this end, we aimed to analyze sRNAs from common bean root hairs (RH), a single-cell model, induced with pure *Rhizobium etli*-Nod factors (NF), a unique type of signal molecule. The sequence analysis of samples from NF-induced and control libraries (3 replicates each) led to identify 132 mature miRNAs, including 63 novel miRNAs. From these, six miRNAs were significantly differentially expressed during NF-induction, including one novel miRNA: miR-RH82. Potential miR-RH82 target members belong to the RNI-like superfamily, encoding an F-box protein containing LRR domain. A parallel degradome analysis of the same samples revealed 29 targets potentially cleaved by novel miRNAs specifically in NF-induced RH samples, however these novel miRNAs were not differentially accumulated in this tissue. Our generated data also allowed us to present the potential production of phasiRNAs in RHs and a catalog of potential miRNA-encoded peptides (miPEPs), suggesting that a considerable number of these molecules could be generated in *P. vulgaris*. This study reveals *Phaseolus vulgaris*-specific novel miRNA candidates and their corresponding targets that meet all criteria to be involved in the regulation of the early nodulation events. Based in our finding of the lack of correlation between very few differentially expressed miRNAs but higher amount of cleaved potential targets in NF-induced RH, we discuss that the miRNA regulation in this tissue seems to be exerted not at the miRNA expression level, but at further down-stream events (*i.e.* loading in AGO1, target cleavage) that would result in a different content of target proteins relevant for the control of early symbiotic stages.

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POSTER 1-20

Role of *Nod* Gene Expression in Competitive Nodule Formation by Clover Rhizobia

Shaun Ferguson, Anthony S. Major, John T. Sullivan, Clive W. Ronson

Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand

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Clovers are an important component of sustainable pastoral systems in New Zealand through their ability to enter a symbiotic relationship with rhizobium bacteria, culminating in the formation of nitrogen-fixing root nodules. Establishment of the symbiosis requires a highly specific and complex signal exchange between the host legume and appropriate rhizobia. The plants exude flavonoids that are perceived by compatible rhizobia through a LysR-type regulator NodD. Activated NodD mediates expression of the nodulation *(nod)* genes, which produce a cocktail of lipochitin oligosaccharide signalling molecules known as Nod factors (NF). Recognition of NF by a compatible legume initiates nodule formation. Different strains of the clover-nodulating species, *Rhizobium leguminosarum* bv. *trifolii* (*Rlt*), vary in their ability to nodulate different clover species effectively, and also to nodulate a particular clover species in competition with other strains. This is important agriculturally as often indigenous strains outcompete added inoculum strains that are better at nitrogen fixation. To determine whether differences in induction of *nod* genes in response to host exudates may contribute to host specificity or competitive ability, we are investigating the induction of *nodA* and *nodF* promoters from four strains that have different host specificities. Initial studies have revealed significant variation in *nod* gene expression in response to the flavonoid 7,4'-dihydroxyflavone. The differences were due to both the host strain background and the particular promoter. We also discovered that most *Rlt* strains have a second, previously unreported, copy of *nodD*, designated *nodD2*, located away from the main *nod* gene cluster. Markerless deletion of *nodD1* from a strain containing a putative *nodD2* revealed only slight reduction in nodulation on various clover species, indicating the second copy may be functional. Future work will determine whether the differences in *nod* and other gene expression in the rhizosphere contribute to host-specificity and competitive ability, and the role of the *nodD2* gene in *nod* gene expression. This will allow us to identify genetic factors contributing to successful symbiosis between rhizobia and clover species, which can be used as selection criteria for high-quality inoculants in New Zealand.

POSTER 1-21

What are the Consequences of the Phosphorylation of PUB1 by Symbiotic Receptors DMI2 and LYK3 in Nodulation?

Mégane Gaston[1], Sylvie Camut[1], Tatiana Vernié[1], Céline Henry[2], Virginie Gasciolli[1] and Christine Hervé[1]

[1] *Laboratoire des Interactions Plantes Micro-organismes (LIPM), INRA 441/CNRS 2594, Castanet Tolosan, France*

[2] *Micalis Institute, INRA, PAPPSO, AgroParisTech, Université Paris-Saclay, Jouy-en-Josas, France*

We identified PUB1, an E3 ubiquitin ligase protein as a negative regulator of rhizobial and AM symbioses in *M. truncatula* (1, 2). We showed that the ubiquitin ligase activity of PUB1 is essential for its negative regulatory function (2). In nodulation, comparison between wild type plants and *pub1* mutant plants, defective in the ubiquitin ligase activity of PUB1, showed an increase in the number of nodules in *pub1* plants (2). We propose a role for PUB1 in the fine-tuning of the root endosymbiotic infection and nodulation and the fungal colonization. PUB1 interacts with two essential symbiotic receptor kinases DMI2 and LYK3 (1, 2). Our studies have suggested that the symbiotic receptors DMI2 and LYK3 are not the targets of the ubiquitylation activity of PUB1 (1, 2). DMI2 and LYK3 are able to phosphorylate PUB1 *in vitro* (1,2), and several phosphosites on PUB1 have been identified by mass spectrometry (C. Hervé and C. Henry, unpublished data). We hypothesize that DMI2 and LYK3 modulate the activity of PUB1 by phosphorylation during symbioses, which then interacts with and ubiquitylates downstream components (currently unknown) in pathways leading to nodulation and/or AM fungal colonization. Thus, our data suggest that LYK3 and DMI2 downstream signaling could be down regulated by a functional PUB1 during the rhizobial and AM symbioses, rather than PUB1 affecting the receptors themselves. Preliminary data to untangle the consequences of the phosphorylation of PUB1 by symbiotic receptors in regulating its targets in nodulation will be presented.

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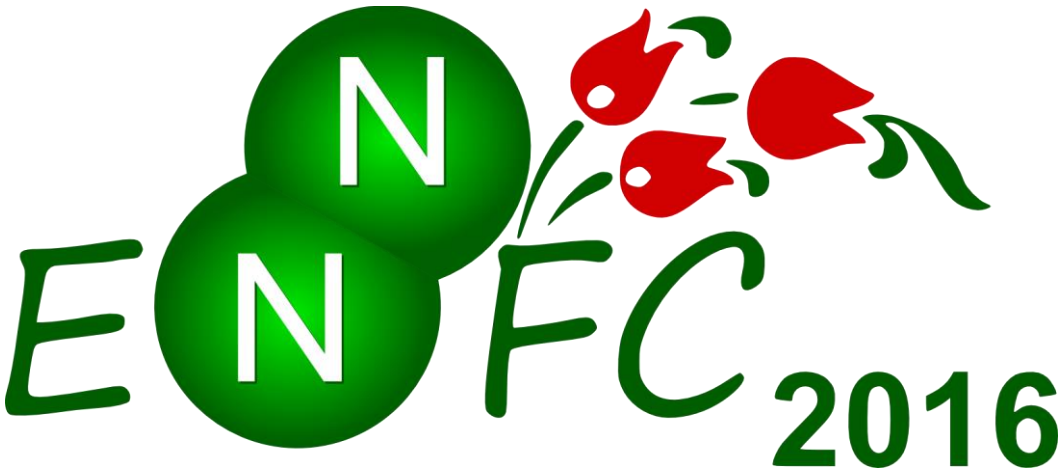

POSTER SESSION 2

Biochemistry of Key Processes and Enzymes

Room Orion

POSTER 2-1 /LIGHTNING TALK/

Comparative Biochemical Studies of *Lotus japonicus* LysM Receptor Like Kinases

Kira Gysel[1,5], M. Vinther[1,5], M. Blaise[1,2,5], J. Cheng[1,5], C. T. Hjuler[1,3,5], Y. Kawaharada[1,5], A. Muszynski[4], M. B. Thygesen[1,3,5], K. R. Andersen[1], S. S. Thirup[1,5], K. J. Jensen[1,3,5], J. Stougaard[1,5]

[1] *Department of Molecular Biology and Genetics - Aarhus University, Denmark*
[2] *Centre d'etudes d'agents Pathogénes et Biotechnologies pour la Santé (CBPS) - Université de Montpellier, France*
[3] *Department of Chemistry, University of Copenhagen, Denmark*
[4] *Complex Carbohydrate Research Center, University of Georgia, United States*
[5] *Centre for Carbohydrate Recognition and Signalling, Aarhus University, Denmark*

Two receptors in the model legume *Lotus japonicus*, NFR1 and NFR5, recognize lipochitooligosaccharides (Nod-factors) secreted by *Mesorhizobium loti*. NFR1 and NFR5 belong to a large family of LysM receptor kinases (LysM-RK) in *Lotus japonicus* containing 3 extracellular LysM domains and an intracellular kinase or pseudokinase domain connected by a short transmembrane domain. LysM-RK are key players in friend-or-foe recognition and are capable of recognizing and distinguishing structurally very similar signaling molecules and initiate appropriate local and systemic responses to those. Additionally, the LysM-RK EPR3 in *L.japonicus* was recently shown to perceive *M.loti* exopolysaccharides (EPS) and regulate bacterial entry (1).

LysM-RK and their ligands are therefore interesting targets for comparative binding affinity studies.

However, biochemical studies of full-length LysM-RK are challenging due to the recalcitrant nature of these single-pass membrane proteins, resulting in laborious recombinant expression, difficult purification and low yields. We developed and optimized an insect cell-based system for recombinant expression of a variety of ligand-binding LysM-RK ectodomains to facilitate purification, obtain higher yields and enable advanced ligand binding assays.

Here, we present the results of binding studies on insect-cell expressed LysM-RK by Microscale thermophoresis and Biolayer interferometry. As an example, the direct binding of the EPR3 ectodomain to *M. loti* EPS, a ligand not containing N-acetyl-D-glucosamine, could be successfully demonstrated with a dissociation constant $K_D = 2.7 \pm 0.2$ µM (1).

References:

[1] *Kawaharada et al. (2009) Nature. 523:308-312*

POSTER 2-2 /LIGHTNING TALK/

Regulation of Bacterial Metabolism by the Phosphotransferase System (PTS^{Ntr})

Carmen Sanchez-Cañizares[1], Francesco Pini[1], Jürgen Prell[2], Philip Poole[1]

[1] *Department of Plant Sciences, University of Oxford. South Parks Road, Oxford, United Kingdom*
[2] *Soil Ecology, RWTH, Aachen, Germany*

The establishment and maintenance of an effective N-fixing symbiosis is intimately interconnected with the metabolism of the plant and requires a complex coupling of biochemical and morphological factors between rhizobia and their host (1, 2). The success of this interaction relies on a fine-tuned coordination of intracellular and extracellular signals, which in the case of the bacterium seems to be exerted by the phosphoenolpyruvate: carbohydrate-phosphotransferase system (PTS). This PTS system is the key signal transduction pathway involved in the regulation of carbon metabolism in Gram-negative and Gram-positive bacteria (3). It acquires the phosphate from phosphoenolpyruvate and passes it through the different components of the system, with the ultimate acceptor being a sugar available in the environment, which is phosphorylated upon transport by the membrane components EIIB and EIIC. In the case of Gram-negative bacteria, PTS^{Ntr} represents an alternative PTS system encoded by the genes *ptsP, npr* and *ptsN*, which preserves the phosphotransfer components, but lacks the permeases, suggesting an exclusively regulatory role (4). Additionally, in *Rhizobium leguminosarum* the *npr* locus is located downstream of *manX*, a gene coding for an $EIIA^{Man}$ homologue. Thus, *npr* seems to be the link between PTS^{Ntr} and the carbohydrate PTS, modulating the intracellular metabolism. In this work we show a differential regulation driven by these systems. While *ptsN* is required for full activation of ABC transport systems and the high affinity K^+ transporter KdpABC (5), *manX* would interact with the TCA cycle. This work shows that mutants on the PTS^{Ntr} system have a reduced transport rate on different nutrients that need an ATP-dependent transporter. However, although the transport rate of *manX* mutants is not affected, they show a reduced growth on several carbon sources and a compromised oxygen consumption when grown on succinate.

The overall aim of this project is to understand how N-fixing bacteria thrive in different nutritional niches adapting their metabolism. This would allow us to engineer a N-fixing bacteria able to improve nitrogen supply to plants for a sustainable crop yield improvement.

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POSTER 2-3 /LIGHTNING TALK/

The Nod Factor Hydrolase of *Medicago truncatula*: An Example of Symbiosis-related Neofunctionalization

Lan-Yue Zhang[1], Jie Cai[1], Wei Liu[1], Ru-Jie Li[1], Kam-Bo Wong[2], Jiangqi Wen[3], Kirankumar S. Mysore[3], Thomas Boller[4], Zhi-Ping Xie[1], Christian Staehelin[1]

[1] *State Key Laboratory of Biocontrol and Guangdong Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-sen University, East Campus, Guangzhou, China*
[2] *Chinese University of Hong Kong, Shatin, Hong Kong, China*
[3] *The Samuel Roberts Noble Foundation, Ardmore, Oklahoma, USA*
[4] *Botanisches Institut der Universität Basel, Zurich-Basel Plant Science Center, Basel, Switzerland*

The symbiotic association between rhizobia and host legumes depends on lipo-chitooligosaccharidic Nod factors. MtNFH1, the Nod factor hydrolase of *Medicago truncatula*, is a specific enzyme that hydrolytically inactivates Nod factors with a C16:2 acyl chain produced by the microsymbiont *Sinorhizobium meliloti* 1021. MtNFH1 is related to class V chitinases (glycoside hydrolase family 18) but lacks chitinase activity, i.e. does not cleave chitin or chitin oligosaccharides. A homology model of the Nod factor-MtNFH1 interaction suggests a substrate binding pocket with a distinct fatty acid binding cleft formed by loops A and B (1). The amino acid sequence of MtCHIT5b is most similar to MtNFH1. The genes are located in tandem on chromosome 4 of *M. truncatula*. MtCHIT5b is a chitinase that efficiently hydrolyzes chitin oligosaccharides but does not cleave Nod factors. Transcript levels of *MtCHIT5b* are elevated in response to inoculation with the fungal pathogen *Fusarium oxysporum*. Hence, MtCHIT5b shows characteristics of classic chitinases involved in plant defense. Substitution of amino acid residues in either loop A or B of MtCHIT5b results in MtCHIT5b variants that show Nod factor cleaving activity. Remarkably, a single serine-to-proline substitution was sufficient to convert MtCHIT5b into a Nod factor cleaving enzyme. Inversely, MtNFH1 with a corresponding reverse substitution lost the capacity to hydrolyze Nod factors (2). These results are in agreement with a substrate-enzyme model that predicts Nod factor cleavage when the C16:2 acyl chain is placed into a distinct fatty acid-binding cleft. To corroborate the symbiotic role of MtNFH1, we currently characterize mutants with *Tnt1* retrotransposon insertions. We identified mutants with reduced Nod factor cleaving activity and a mutant completely defective in *MtNFH1* expression. Work is ongoing to characterize their symbiotic phenotypes. In summary, our findings support the view that *MtNFH1* evolved from an ancestral *MtCHIT5b* variant by gene duplication and subsequent neofunctionalization. In other words, plant defense related elements were exploited for symbiotic interactions.

References:

[1] *Tian et. al. (2013) Plant Physiol. 163:1179-1190.*
[2] *Zhang et al. (2016) submitted.*

POSTER 2-4 /LIGHTNING TALK/

From Symbiosis to Biotechnology: The Metal Ion-inducible Autocleavage (MIIA) Domain

Jana Schirrmeister[1], Susan Ibe[1], Elisabeth Hoyer[1], Martina Liebmann[1], Susanne Zehner[1], Karim Fahmy[2], Michael Göttfert[1]

[1] *Institute of Genetics, Technische Universität Dresden, Dresden, Germany*
[2] *Institute of Resource Ecology, Helmholtz-Zentrum Dresden-Rossendorf, Dresden, Germany*

Bradyrhizobium japonicum is a Gram-negative soil bacterium and the symbiont of several legumes, e.g. soybean. The plant signal genistein leads to the activation of more than 100 genes. One induced gene cluster encodes a type III secretion system. This is used to transfer effector proteins into the plant cell. The nodulation outer protein NopE1 was proven to be one of these effector proteins (1,2). Bioinformatics analyses revealed that it contains two domains of unknown function (DUF1521), each encompassing about 170 amino acids. NopE1 was expressed in *Escherichia coli* and purified. The protein is cleaved in the presence of calcium. The cleavage site is within the DUF1521 domain, for which we now use the term "metal ion-inducible autocleavage" (MIIA) domain (3,4).

Blast searches unveiled that the MIIA domain is conserved not only in proteins from *B. japonicum* strains but also in proteins of various α-, β-, γ- and δ-Proteobacteria, e.g. the plant growth-promoting endophyte *Burkholderia phytofirmans* PsJN or the coral pathogen *Vibrio coralliilyticus* ATCC-BAA450. The putative protein Vic_001052 from *V. coralliilyticus* contains one MIIA domain. This MIIA domain shows self-cleavage not only in the presence of calcium ions but also in the presence of manganese but not with magnesium ions (4).

Based on the properties of the MIIA domain, we used it as a self-cleaving protein linker. In biotechnology, proteins are often expressed and purified as fusion proteins, which are later cleaved by a costly protease. In contrast, the MIIA domain is an easy low cost tool to release the protein from the fusion partner. Initial tests indicate that cleavage within the MIIA domain is not influenced by the fusion partner. Cleavage is accomplished within minutes on ice and at moderate temperatures. Cleavage is also tolerant towards a pH range from about 5 to 9 (5).

For a more detailed structural characterisation of the MIIA domain, circular dichroism (CD) spectroscopy was used. The MIIA domain is largely unstructured with random coils covering about 75 % of the protein. Upon addition of calcium ions, the percentage of α-helices and β-sheets is increasing. Fluorescence spectroscopy also indicates a strong influence of calcium ions on protein structure.

References:

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POSTER 2-5 /LIGHTNING TALK/

Heterologous Expression of Enzymes of the Nitrogenase Pathway

Laure Decamps, Ivana Djurdjevic, Oliver Einsle

Institute of Biochemistry, Albert-Ludwigs-Universität, Freiburg-im-Breisgau, Germany

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Nitrogenase is the only enzyme able to catalyze the reduction of dinitrogen (N_2) to ammonium (NH_3^+) and is therefore essential to fix the nitrogen present in the atmosphere. However, it is not present in eukaryotic organisms, but only in some bacteria and archaea. Hence, plants must rely on interactions with these prokaryotes to obtain nitrogen. Nitrogen availability being a limiting factor for plant growth, making plants independent from these interactions would be a major biotechnological breakthrough.

The transfer of the nitrogen fixation machinery from free-living diazotrophs to model bacteria represents the first step of such a development. We aim to express genes of the nitrogenase pathway from model diazotrophs, for which the lack of genetic tools stands in the way of enzyme bioengineering, to well-characterized laboratory strains. A "minimal set" of 6 genes encoding structural and biosynthetic components (*nifB, nifE, nifN, nifH, nifD, nifK*) has been proposed (1), however so far no nitrogen fixation could be achieved in a heterologous organism with less than 9 genes (the "minimal set" plus *nifX*, *nifV*, and *hesA*) of *Paenibacillus* sp. WLY78 (2). Furthermore, the additional expression of some other accessory proteins may improve the expression and the activity of these enzymes. We intend to first identify the enzymes from various diazotrophs that can be expressed and active in a heterologous organism, then combine them to optimize the nitrogen fixation efficiency. In parallel, we are characterizing these enzymes and setting up reaction assays *in vitro*. This will allow us to engineer these enzymes to increase their activity *in vitro* and *in vivo*.

We have successfully expressed the different components of nitrogenase and of the nitrogenase maturation pathway from a soil free-living organism in a laboratory strain of *Escherichia coli* and combined the expression of some of these components by building new vectors. In parallel to activity assays *in vitro*, we undertook interaction studies to assess the role of accessory proteins that remain undescribed yet.

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POSTER 2-6 /LIGHTNING TALK/

Exploring the Function of the Inorganic Phosphate Transporter (PiT)-associated Protein in *Sinorhizobium meliloti*

Daniel K. C. Hsieh[1], Vahid Hosseininaveh[2], Rahat Zaheer, Richard Morton[1] and Turlough M. Finan[1]

[1] *Department of Biology, McMaster University, Hamilton, Ontario, Canada*
[2] *Department of Plant Protection, University of Tehran, Karaj, Iran*

Phosphate (Pi) is one of the most important macronutrients required for cellular functions such as signal transduction, membrane lipid dynamics, and nucleotide synthesis. Phosphate is often a limiting nutrient in the environment and its availability can determine the rate of growth of plants and microbes. In *Sinorhizobium meliloti*, there are three Pi transport systems, one of which is a Pit-like transporter, homologous to the well characterized *E. coli* PitA phosphate transporter. However, unlike in *E. coli*, *S. meliloti pit* co-occurs in an operon with *pap* (*orfA*), which encodes a hypothetical protein of unknown function. Previous work has shown that the *pap-pit* operon is negatively regulated by the response regulator PhoB and is therefore expressed under conditions of excess inorganic phosphate. Here, the function of *pap* or *pit-accessory protein (pap*) is investigated through a bioinformatics and mutagenesis approach. Pap orthologs analyzed from bacterial genomes reveal several highly conserved residues within putative PhoU-like motifs. When *S. meliloti* Pap-Pit is the sole phosphate transporter in *S. meliloti* and *E. coli*, substitutions of several conserved residues result in a growth defect in minimal media with inorganic phosphate as the only source of phosphorus. Comparative analyses of the putative Pap-like structures revealed a six alpha-helical bundle similar to that of PhoU, suggesting that Pap may function as a positive regulator of phosphate uptake through the Pit system.

POSTER 2-7

A Regulatory Model for Acid-induction of the *lpiA/acvB* Operon in *Ensifer medicae*

Rui Tian[1], Stephan Heiden[1], Wan Adnawani[1], Julie Ardley[1], Giovanni Garau[2], Euan K. James[3], Rekha Seshadri[4], Nikos Kyrpides[4] and Wayne Reeve[1]

[1] *Centre for Rhizobium Studies, Murdoch University, Murdoch, Australia*
[2] *Dipartimento di Scienze Ambientali Agrarie e Biotecnologie Agro-Alimentari, University of Sassari, Sassari, Italy*
[3] *The James Hutton Institute, Invergowrie, Dundee, Scotland, United Kingdom*
[4] *DOE Joint Genome Institute, Walnut Creek, California, United States*

Ensifer medicae strains are generally more acid-tolerant than *E. meliloti* strains, and are symbiotically associated with *Medicago* species that are adapted to moderately acid soils (1). In the acid-tolerant model strain *E. medicae* WSM419, one of the critical determinants that enable survival in lethal acidic conditions is the acid-activated expression of the low-pH-induced gene *lpiA* (Smed_5951), which encodes a putative lysylphosphatidylglycerol (LPG) synthase. The *lpiA* gene is immediately upstream of the *virJ* homolog *acvB* (Smed_5950), which encodes a putative alpha/beta-hydrolase that is hypothesized to remove lysine from LPG. Expression of both *lpiA* and *acvB* is dependent on FsrR (fused sensor regulator), however a 3-fold acid induction of *lpiA* in an *fsr*R (Smed_5952) knockout background still remains (2).

We have now identified a complete regulatory circuit for the acid activation of the *lpiA*/*acvB* operon in *E. medicae*. In WSM419, genes in this regulatory circuit include *ebpA* (Smed_5956) (an enhancer binding protein), *tcsA* (Smed_5954)/*tcrA* (Smed_5953) (a two component sensor/regulator) and *rpoN* (Smed_0015). The intergenic region upstream of the *lpiA*/*acvB* operon contains a putative RpoN (RNA polymerase sigma factor for nitrogen metabolism) binding motif. The transcription start site (TSS) for *lpiA* and *acvB* was RACE mapped to 14 bases downstream of the predicted RpoN binding site. Knockout mutations in *ebpA*, *tcrA*, *tcsA* and *acvB* did not compromise nitrogen fixation with *Medicago murex*, *M. polymorpha*, *M. sativa* or *M. truncatula*, whereas *Medicago* hosts inoculated with the *rpoN* mutant had white, ineffective nodules. Expression of a plasmid-borne *lpiA-gusA* fusion in each of the regulatory mutants showed that TcsA/TcrA, RpoN and EbpA were essential for acid activation of *lpiA*/*acvB*. The *tcsA*/*tcrA*/*fsr*R regulatory genes are present and required for the acid activation of the *lpiA*/*acvB* operon in 18 studied *E. medicae* strains.

Analysis of sequenced genomes shows that, in most *Ensifer* species, the *lpiA*/*acvB* operon is located on the chromosome and lacks an RpoN binding site upstream of *lpiA*. *E. meliloti* strains completely lack the *ebpA*, *tcrA*, *tcsA* and *fsr*R regulatory loci, except for *E. meliloti* Mlalz-1, which has acquired a plasmid containing a homolog of *lpiA*/*acvB* along with regulatory loci. All sequenced *E. medicae* genomes, except for strain Di28, have the regulatory and *lpiA*/*acvB* loci co-located on a pSymA-type symbiotic plasmid. Here we provide a model of this unique regulatory system that governs expression of the *lpiA*/*acvB* operon in *E. medicae*.

References:

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[2] *Reeve et al. (2006) Microbiology 152: 3049–3059.*

POSTER 2-8

An Intriguing Mode of Pantothenate Synthesis in Rhizobia

Mariana L. Sámano[1], T. V. Toledo[2], A. Dávalos[1] and A. G. Santos[1]

[1] *Center for Genomics Sciences, UNAM. Av. Universidad s/n Col. Chamilpa, Cuernavaca Morelos, México*

[2] *Biotechnology Institute UNAM, Av. Universidad s/n Col. Chamilpa, Cuernavaca Morelos, México*

Pantothenate (vitamine B_5) is the metabolic precursor of coenzyme A (CoA) an essential cofactor of a large number of enzymes. Pantothenate is synthesized from two precursors, pantoate and beta-alanine. Seminal studies in enterobacteria stated that beta-alanine is produced in a sole step by decarboxylation of L-aspartate in a reaction catalyzed by the aspartate decarboxylase enzyme (ADC) encoded in the *panD* gene. Recent studies in the archaeon *Thermococcus kodakarensis* revealed that in the absence of an ADC homolog beta-alanine is synthesized from a glutamate decarboxylase (GDC) homolog able to decarboxylate both glutamate and aspartate [1].

ADC homologues are absent from most α-proteobacteria belonging to the order Rhizobiales. Since several reference strains are pantothenate prototrophs, we hypothesized that an unknown enzyme with aspartate decarboxylase activity is involved in beta alanine synthesis in these bacteria. As part of our experimental strategy to identify this enzyme, the genomic library of *R. etli* CFN42 was mobilized by conjugation to an *E. coli ⊿panD* mutant auxotroph of beta-alanine. The *⊿panD* transconjugants rescued from a beta-alanine/pantothenate-free chemical defined medium, acquired a 20 Kb chromosomal fragment from the R. etli genomic library and recovered the beta alanine prototrophy. The subcloning and genetic complementation analysis revealed that a fragment of 2 Kb, containing the gene encoding for glutaminase A (*glsA*) of *R. etli*, was sufficient to recover the beta alanine prototrophy to the ΔpanD mutant. Reports in the literature have shown the existence of "moonlighting proteins" that perform more than one unrelated function in analogy to moonlighting people who have multiple jobs. Studies *in vitro* with the glutaminase of E. coli revealed that this enzyme is also able to decarboxylate glutamate [2].

Based on these evidence, we are testing the hypothesis that glutaminase A of *R. etli* is a bifunctional enzyme that can hydrolyze glutamine and decarboxylates aspartate. These data may represent the first alternative model of beta alanine synthesis in bacteria.

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POSTER 2-9

Expression of a Functional Oxygen-labile Nitrogenase Component in the Mitocondrial Matrix of Aerobically Grown Yeast

Gema López-Torrejón[1], Emilio Jiménez-Vicente[1], José María Buesa Galiano[1], Jose A. Hernandez[2], Hemant K. Verma[1] and Luis M. Rubio[1]

[1] *Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid, Pozuelo de Alarcón, Madrid, Spain*
[2] *Department of Biochemistry, Midwestern University, Glendale, United States*

The extreme sensitivity of nitrogenase towards oxygen stands as a major barrier to engineer biological nitrogen fixation into cereal crops by direct nif gene transfer (1, 2). Here, we use yeast as a model of eukaryotic cell and show that aerobically grown cells express active nitrogenase Fe protein when the NifH polypeptide is targeted to the mitochondrial matrix together with the NifM maturase. Co-expression of NifH and NifM with nif-specific Fe-S cluster biosynthetic proteins NifU and NifS is not required for Fe protein activity, demonstrating NifH ability to incorporate endogenous mitochondrial Fe-S clusters. In contrast, expression of active Fe protein in the cytosol requires both anoxic growth conditions and co-expression of NifH and NifM with NifU and NifS. Our results show the convenience of using mitochondria to host nitrogenase components, thus providing instrumental technology for the grand challenge of engineering N2-fixing cereals (3).

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POSTER 2-10

Regulation of Polyhydroxybutyrate Synthesis in *Bradyrhizobium diazoefficiens*

Juan Ignacio Quelas[1], Socorro Mesa[2], Elias Mongiardini[1], Dieter Jendrossek[3], Anibal R. Lodeiro[1]

[1] *IBBM, UNLP-CONICET, Argentina*
[2] *EEZ-CSIC, Spain*
[3] *University Stuttgart, Germany*

Polyhydroxyalkanoates (PHA) are carbon and energy reserve polymers that accumulate in various prokaryotic species. Microorganisms synthesize and store PHA inside their cytoplasm as carbon and energy reserves when the carbon source is not limiting. There are several types of this polymer, being poly-3-hydroxybutyrate (P3HB or PHB) the most common.

In this work, we determined that, when grown with mannitol as the sole carbon source, *Bradyrhizobium diazoefficiens* produces a homopolymer composed only of 3-hydroxybutyrate units (PHB). Conditions of oxygen limitation (such as microoxia, oxic stationary phase and bacteroids inside legume nodules) were permissive for the synthesis of PHB, which was observed as cytoplasmic granules. To study the regulation of PHB synthesis in *B. diazoefficiens*, we generated mutants in the regulator gene *pha*R and the phasin genes *phaP1* and *phaP4*. Under permissive conditions, mutation of *pha*R impaired PHB accumulation, and a *phaP1/phaP4* double mutant produced around three times more PHB than the wildtype. Furthermore, PHB was accumulated in a single, large cytoplasmic granule in the *phaP1/phaP4* mutant. In addition, PhaR behaved as a global regulator, because it repressed the expression of several PHB biosynthetic genes such as *phaP1*, *phaP4*, *phaA1*, *phaA2* (3-ketoacyl-CoA thiolase), *phaC1*, *phaC2* (PHB synthases), as well as that of the CRP/FNR-type transcription regulator *fixK*$_2$, which in turn positively regulates the expression of more than 200 genes related with the microoxic lifestyle. Accordingly, the *pha*R mutant not only produced less PHB, but also overproduced extracellular polysaccharides (EPS).

In addition to the effects observed in the free-living bacterial state, in the symbiotic state *pha*R mutants promoted higher soybean shoot dry weight and competitiveness for nodulation than the wildtype. These effects were contrary to that of *phaC1* mutant strains, which are also defective in PHB synthesis, and indicate that these effects are not due solely to defects in PHB accumulation.

These results suggest that *pha*R not only regulates PHB granules formation by controlling the expression of phasins and biosynthetic enzymes, but also acts as global regulator of excess carbon allocation by controlling *fixK*$_2$, which may also regulate several aspects of the symbiosis with soybean plants.

POSTER 2-11

Review of Studies on Four Enzymes in Bacteriochlorophyll (*BChl*) and Chlorophyll (*Chl*) Biosynthesis

Silvio M. D. Nascimento, Yuanyuan Zou, Qi Cheng

Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China
chengqi@caas.cn

We summarize the progress of developing methods which bring us the information to understand the mechanism of Chlorophyll biosynthesis emphasizing four enzymes in its last biosynthesizing steps. The biosynthesis of Chlorophyll (*Chl*) /Bacteriochlorophyll (*Bchl*) is essential for the occurrence of photosynthesis. These four different enzymes catalyze some of the last chemical reactions steps in the *Bchl*/*Chl* biosynthesis, and they are: the Light-Dependent Protochlorophyllide (*Pchlide*) Oxidoreductase (*LPOR:* EC 1.3.1.33), the Light-Independent *Pchlide* oxidoreductase (*DPOR*: EC 1.3.7.7), *Chlide* Reductase (*COR*: EC 1.3.99.35) and the Divinyl Reductase (*DVR*: EC 1.3.1.75). The Light-Dependent Protochlorophyllide (*Pchlide*) Oxidoreductase (*LPOR:* EC 1.3.1.33) and the Light-Independent *Pchlide* oxidoreductase (*DPOR*: EC 1.3.7.7) are responsible for the reduction of the C_{17}=C_{18} double ring D in the tetrapyrrole configuration of *Pchlide* to form *Chlide*. *Chlide* Reductase (*COR*: EC 1.3.99.35) is responsible for the reduction of C_7=C_8 in the ring B (*Bchl*). The Divinyl Reductase (*DVR*: EC 1.3.1.75) reduces the C_8 vinyl group in *8-vinyl-Chlidea* to produce *Chlidea* in the Bacteriochlorophyll biosynthesis. In this review, the most important discoveries related to the mechanisms, structures and interactions of these four enzymes, that were made so far, are presented, and the sequence alignment, phylogenetic and molecular evolutionary analysis of these four enzymes are conducted.

Keywords: Photosynthesis Chlorophyll; Bacteriochlorophyll Biosynthesis; Enzymes DVR, DPOR, LPOR and COR.

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POSTER 2-12

Some Studies on the Regulation of Glutamate Dehydrogenase with Mutants of *Azospirillum brasilense*

Pushpita Maulik

Department of Chemical Engineering, Indian Institute of Technology, Kharagpur India

Ammonia assimilation in the free-living diazotroph Azospirillum brasilense is carried out by glutamate dehydrogenase(GDH) and glutamine synthetase(GS)/glutamate synthase pathways. This work was started with the intention of studying the regulation of GDH synthesis in this bacterium. GDH level in *A.brasilense* grown in succinate minimal medium with high ammonia is ten-fold higher than those in cells grown with low ammonia or amino acids glutamine, glutamate, arginine, proline. The nitrogen regulatory (Ntr) system for the overall regulation of N-metabolism, is present in *A.brasilense* but here the organization of ntr genes are different from that of enteric bacteria. Of the ammonia assimilatory enzymes, regulation of glutamine synthetase (GS) has been studied extensively in enteric bacteria, in *A.brasilense* and other diazotrophs. Recent works on enteric bacteria show that GDH regulation is also quite complicated. However, regulation of GDH synthesis in *A.brasilense* remains poorly understood. The enzymatic properties of GDH in *A.brasilense* has been characterized earlier – this enzyme is cold-labile, has dual coenzyme specificity (for NADPH& NADH), and has striking kinetic properties.

Mutants of *A.brasilense* were isolated; these mutants were isolated longtime back by chemical mutagenesis using nitroso guanidine, and selected on the basis of Nif - phenotype - minute colonies that grow on succinate minimal medium containing 0.003% of yeast extract as sole N-source, were replica-plated on NH4+ plus and minus medium and later assayed for nitrogenase. No nitrogenase was detected.

Physiological characterization of these mutants revealed that there were two types of mutants : type-I where GDH syn was not induced and was low even when the cells were grown with high ammonia; GS remained adenylylated even under low NH4+ condition. This mutant did not grow on amino acids glutamine, glutamate and nitrate; type-II - these mutants showed constitutively high GDH level in cells grown in high and low ammonia; GS levels were low under both the above conditions but the adenylylation was normal. The mutants showed slow growth on glutamate, glutamine, arg, prol and nitrate. When glutamate was the N-source, type II cells showed osmosensitivity.

Biochemical studies of GDH from type-II mutant showed that unlike the enzyme from wildtype cells (wt enzyme), this enzyme was not cold-labile and has specificity for only NADPH. GDH from type-I mutant had similar properties as the wt enzyme.

GDH from the type-II mutants was purified (80-fold) by ammoniumsulphate precipitation, gel-filtration chromatography, affinity chromatography on Redsepharose, and the kinetic properties studied. The km of this enzyme for 2-oxo-glutarate, NH4Cl and glutamate were different from those of the wt enzyme. The molecular weight of the enzymes were same. It appears that this enzyme could be a second GDH being transcribed from a different site on the genome. How the regulatory defect is suppressing the normal GDH synthesis and producing the second GDH is not understood.

Thus here two types of regulatory defects are seen, and in both cases GDH is affected along with the changes in general N-metabolism. More detailed genetic studies with transposon mutagenesis and gene fusion techniques may lead to better understanding.

Keywords: *Azospirillum brasilense,* glutamate, dehydrogease, N-assimilation, regulatory mutants.

POSTER 2-13

Structural and Biochemical Characterisation of the *Lotus japonicus* LYS6: A LysM Receptor-like Kinase Involved in Chitin Perception

Jeryl Cheng[1], Kira Gysel, Maria Vinther Zoltán Bozsóki, Simona Radutoiu, Søren Thirup, Jens Stougaard, Mickaël Blaise[2]

[1] *Department of Molecular Biology and Genetics, Centre for Carbohydrate Recognition and Signalling, Aarhus University, Denmark*
[2] *CR1-CNRS, Centre d'études d'agents Pathogènes et Biotechnologies pour la Santé, Université de Montpellier, France*
jeryl@mbg.au.dk

Many species of plants associate with a wide range of microorganisms to form symbiotic relationships, trading carbon sources in exchange for nitrogen or phosphate. The success of this relationship hinges on the plants' ability to recognize beneficial organisms to promote association and to limit the intrusion of pathogens. Nodulation factors (NFs) are decorated chitin oligosaccharides secreted by rhizobia. NFs are recognized by related plant LysM receptor kinases in legumes to trigger the nitrogen-fixing symbiosis pathway. LysM receptors may also perceive other chitin molecules such as Myc factors (symbiotic), and pathogen-associated molecular patterns (PAMP) from fungus and bacteria which trigger the defence pathway. In *Lotus japonicus*, there are 17 different LysM receptor like kinase (LysM-RLKs) genes encoded in the genome. Amongst them, NFR1 (Active Kinase) and NFR5 (Inactive Kinase) had been shown to bind NF and are key players in the nodulation/symbiosis pathway in *L. japonicus*. However, the functions of the other LysM-RLKs in *L. japonicus* have not been determined. We aim to characterize the *lys6* gene. As it is ubiquitously expressed in *L. japonicus*, it is a good candidate gene that might be involved in plant defence. We have established a protocol to express and purify LYS6 ectodomain. We have also crystallized and solved the structure of the Lys6 ectodomain at 2.1Å resolution. Additionally, the binding affinity of Lys6 ectodomain to various chitin-based molecules has been determined using Microscale Thermophoresis. Our *in vivo* biochemical data strongly suggests that Lys6 is a PAMP receptor involved in chitin perception.

POSTER 2-14

The Nodule-specific Signal Peptide Peptidase is Required for Nitrogen Fixing Bacteroid Differentiation in *Medicago truncatula*

Rui Lima, Attila Farkas, Attila Szűcs, Edit Ábrahám, Edina Kiss, Hilda Tiricz, Olga Nagy, Attila Kereszt, Éva Kondorosi

Institute of Biochemistry, Biological Research Centre HAS, Szeged, Hungary

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The signal peptide peptidase (SPP) is a conserved intramembrane aspartyl protease which processes signal peptides (SP) arising from cleavage of secretory preproteins in the endoplasmic reticulum (ER) by the signal peptidase (1). SPP can clean ER from the remnant signal peptides but it can also liberate small biologically active oligopeptides from them.

In *Medicago truncatula* root nodules, several hundreds of secreted nodule specific cysteine-rich (NCR) peptides are produced in the symbiotic cells (2). The signal peptides of the NCRs are relatively conserved unlike the highly diverse mature NCR peptides (3). As NCRs are produced in large amounts all along the differentiation of symbiotic cells, removal of their signal peptides from the ER might be essential for normal cell functioning. On the other hand it cannot be excluded that conservation of the NCR signal peptides is maintained for generation of SP-derived oligopeptides with biological activity. One of the two *Medicago* SPPs is highly nodule-specific, thus it is plausible that its function could be important in maturation of symbiotic cells and development of fully functioning nitrogen-fixing bacteroids via processing of the NCR signal peptides.

We created several independent stable transgenic lines with down-regulation of the nodule-specific (ns) *SPP* with RNA-interference (RNAi). In these lines the nsSPP mRNA levels were reduced to 3-10% of the wild type and the nodules were small and Fix$^-$, lacking nitrogenase activity and functional nitrogen-fixing zone (ZIII). The bacteroids were slightly elongated in the interzone (IZ), but their further differentiation was aborted in line with their transcriptome analysis. Thus, the strong phenotype of these RNAi lines confirms an important role for nsSPP in terminal bacteroid differentiation.

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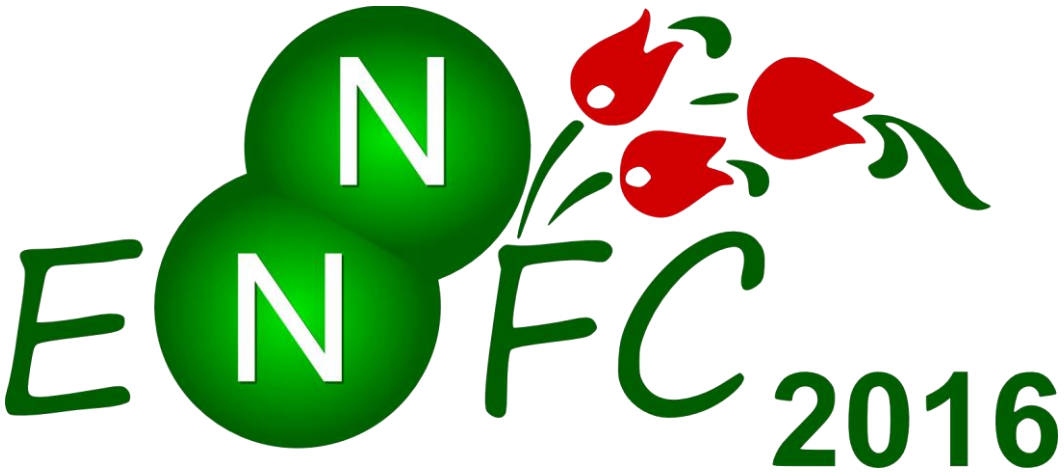

POSTER SESSION 3

Infection and Invasion

Room Orion

POSTER 3-1 /LIGHTNING TALK/

NF-Y TFs as Key Regulators of Nodule Development and Infection

Arnaud Bethencourt, Agnès Lepage, Farid Regad, Carolina Rípodas, Maël Baudin*, Lisa Frances, Pascal Gamas, Fernanda de Carvalho-Niebel and Andreas Niebel

Laboratoire des Interactions Plantes-Microorganismes (LIPM), CNRS, UMR2594, Castanet-Tolosan, France *andreas.niebel@toulouse.inra.fr*

* *Present address: Department of Plant and Microbial Biology, University of California, Berkeley, California, United States*

Plants belonging to the legume family are able to interact symbiotically with nitrogen fixing bacteria named rhizobia. This symbiotic interaction leads to the formation of a new organ on the roots of the host plants, called the root nodule, inside which atmospheric nitrogen is fixed for the benefit of the plant. Evidence for a key role for NF-Y transcription factors (TFs) during symbiotic infection and legume nodule development has gradually emerged (Combier et al., 2006; Zanetti et al., 2010; Soyano et al., 2013; Laloum et al., 2014; Laporte et al., 2014; Xiao et al., 2014; Baudin et al., 2015). NF-Y are CCAAT-box binding TFs also called nuclear factor Y (NF-Y) (Laloum et al., 2013). The heterotrimeric NF-Y is composed of the DNA-binding subunit NF-YA associated with two histone-like subunits NF-YB and NF-YC. We are trying to understand the mechanisms by which NF-Y TFs control nodule development and infection. We have shown that *MtNFYA1*, *MtNF-YB16, and MtNF-YC2*, form the main NF-Y trimer, active during nodulation in *Medicago truncatula.*

We will present data on the identification and characterization of their target genes using ChIP-seq and RNA-seq and also their interacting proteins partners.

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POSTER 3-2 /LIGHTNING TALK/

SCARN a Novel Class of SCAR Protein that is Required for Root-hair Infection During Legume Nodulation

Liping Qiu[1], Jie-shun Lin[1], Ji Xu[1], Shusei Sato[2,3], Martin Parniske[4], Trevor L. Wang[5], J. Allan Downie[5], Fang Xie[1]

[1] *National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai, Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China*
[2] *Kazusa DNA Research Institute, Kisarazu, Japan*
[3] *Graduate School of Life Sciences, Tohoku University, Sendai, Japan*
[4] *University of Munich LMU, Faculty of Biology, Martinsried, Germany*
[5] *John Innes Centre, Norwich, United Kingdom*

Rhizobial infection of legume root hairs requires a rearrangement of the actin cytoskeleton to enable the establishment of plant-made infection structures called infection threads. In the SCAR/WAVE (Suppressor of cAMP receptor defect/WASP family verpolin homologous protein) actin regulatory complex, the conserved N-terminal domains of SCAR proteins interact with other components of the SCAR/WAVE complex. The conserved C-terminal domains of SCAR proteins bind to and activate the actin-related protein 2/3 (ARP2/3) complex, which can bind to actin filaments catalyzing new actin filament formation by nucleating actin branching. We have identified, SCARN (SCAR-Nodulation), a gene required for root hair infection of *Lotus japonicus* by *Mesorhizobium loti*. Although the SCARN protein is related to Arabidopsis thaliana SCAR2 and SCAR4, it belongs to a distinct legume-sub clade. We identified other SCARN-like proteins in legumes and phylogeny analyses suggested that SCARN may have arisen from gene duplication and acquired specialized functions in root nodule symbiosis. Mutation of SCARN reduced formation of infection threads and their extension into the root cortex and slightly reduced root-hair length. However we observed no effect of *scarn* mutations on trichome development or on the early actin cytoskeletal accumulation that is normally seen in root hair tips shortly after *M. loti* inoculation, distinguishing them from other symbiosis mutations affecting actin nucleation. The C-terminal domain of SCARN binds to ARPC3 and ectopic expression of the N-terminal SCAR-homology domain (but not the full length protein) inhibited nodulation. In addition, we found that SCARN expression is enhanced by *M. loti* in epidermal cells and that this is directly regulated by the NODULE INCEPTION (NIN) transcription factor.

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POSTER 3-3 /LIGHTNING TALK/

Host Cell Reprogramming for Rhizobial Root Infection

A. Kelner[1], L. Frances[1], M. R. Cerri[2], M. Beck[1], M. F. Guerrero-Molina[1], M. Chabaud[1], D. G. Barker[1], G. E. Oldroyd[3], J. Fournier[1] and F. de Carvalho-Niebel[1]

[1] *Laboratory of Plant Microbe Interactions (LIPM), INRA, CNRS, Université de Toulouse, Castanet-Tolosan, France*
[2] *Current address: Faculty of Biology, University of Munich (LMU), Institute of Genetics, Grosshaderner Str. 2-4, D-82152 Martinsried, Germany*
[3] *John Innes Centre, Norwich NR4 7UH, United Kingdom*

The development of a functional nitrogen-fixing nodule in legumes depends on a successful molecular dialogue between the plant and respective rhizobial partner before the bacteria can enter the host root. Rhizobial infection then occurs *via* a host defined pathway within newly formed apoplastic tubular structures (infection threads), which involves coordinated reprogramming of adjacent root cell layers. We recently demonstrated that two closely-related ERF-type transcription factors (ERN1 and ERN2) are essential for early rhizobial infection of *Medicago truncatula* roots (1,2,4). While *ERN1* and *ERN2* act in concert to regulate early steps of root hair epidermal cell entry, only ERN1 is essential for root cortical cell infection. In addition to genetic approaches, *in vivo* cell imaging has revealed that ERN1 sequentially accumulates in the nuclei of root cells undergoing infection (3), which underlines the importance of cell-specific reprogramming during these early steps of bacterial entry. We now focus on the understanding of the nuclear reprogramming that takes place at this particular early stage of infection by using a variety of complementary strategies. This includes the analyses of gene expression changes in single and double mutant lines to identify downstream genes directly dependent on both ERN1/ERN2 factors and also new *in vivo* cell imaging approaches combined with symbiotic mutants that allow us to follow precise nuclear changes that occur during this early symbiotic stage.

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POSTER 3-4 /LIGHTNING TALK/

The ERF Required for Nodulation1 (ERN1) Transcription Factor is Required for Root Nodule Infection in *Lotus japonicus*

Yasuyuki Kawaharada[1,2], Simon Kelly[1,2], Niels Sandal[1,2], Euan K. James[3], Simona Radutoiu[1,2] and Jens Stougaard[1,2]

[1] *Centre for Carbohydrate Recognition and Signalling, Aarhus University, Denmark*
[2] *Department of Molecular Biology and Genetics, Aarhus University, Denmark*
[3] *The James Hutton Institute, Invergowrie, United Kingdom*

The symbiotic association between legume and rhizobia is accomplished through two developmental processes: The rhizobial infection and nodule organogenesis. The final outcome of the interaction is the formation of root nodules within which the rhizobia reside. Several legume transcription factors have been identified and shown to be important for this symbiosis. For example, ERF Required for Nodulation (ERN)1, ERN2 and ERN3 are transcription factors containing an APETALA2/Ethylene Responsive Factor (AP2/ERF) domain, that were identified in *Medicago truncatula*. In this study, we characterise the role of ERNs in the determinate nodule forming model legume *Lotus japonicus,* and identify factors involved up- and down-stream of these transcription factors.

Ern1 and *Ern3* homologs have been identified in the current *L. japonicus* genomic sequence data (v3.0), but no *Ern2* homolog could be identified. Four *ern1* allelic mutants containing LORE1 retrotransposon insertions, show are impaired in infection thread formation when inoculated with the symbiotic bacteria *Mesorhizobium loti*. However, it was observed that they form effective nodules via a crack-entry colonisation mechanism 3 to 4 weeks post-inoculation. The nodules formed, *ern1* mutants exhibited abnormal and peg-type cortical infection threads. These observations suggest that ERN1 is important for forming infection threads in both root hairs and nodules. Spontaneous nodulation assays using autoactive CCaMK (T265D) transgenic roots and exogenous cytokinin treatment, revealed that ERN1 additionally regulates nodule organogenesis. RT-qPCR and *Ern1* promoter GUS analyses revealed that *Ern1* expression is induced by Nod factor, and this expression is localised to the susceptible zone of the epidermis at early stages of symbiosis and in the cortex at later stages during development of nodule primordia. In addition, we have identified that the *Epr3* encoded Expolysaccharide receptor[1], and several other genes are induced directly or indirectly by ERN1[2,3].

Results that position the ERN1 transcription factor in the *Lotus* symbiotic signalling genetic pathway will be presented and discussed.

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POSTER 3-5 /LIGHTNING TALK/

Regulation of *Lotus japonicus ERN1* by the CCaMK/CYCLOPS Complex Constitutes a Central Step in the Transcription Factor Network Controlling Bacterial Accommodation

Marion R. Cerri[1], Quanhui Wang[2], Xiaolin Li[2], Paul Stolz[1], Jessica Folgmann[1], Lisa Frances[3], Katja Katzer[1], Fernanda de Carvalho-Niebel[3], Trevor L. Wang[4], Allan Downie[4], Fang Xie[2*] and Martin Parniske[1]

[1] *Faculty of Biology, University of Munich (LMU), Institute of Genetics, Martinsried, Germany*
[2] *National Key Laboratory of Plant Molecular Genetics, CAS Center for Excellence in Molecular Plant Sciences, Institute of Plant physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China*
[3] *Laboratory of Plant-Microbe Interactions (LIPM), Centre National de la Recherche Scientifique (CNRS, UMR2594), Institut National de la Recherche Scientifique (INRA), Castanet-Tolosan, France*
[4] *John Innes Centre, Norwich, United Kingdom*

The nitrogen-fixing root nodule symbiosis of legumes contributes significantly to protein nutrition of humans and animals. The mechanism of bacterial entry into plant cells and the regulation of this process is a key feature of this interaction. In order to study the genetic basis of the infection process, a forward genetic screen was performed in search for infection mutants of the model legume *Lotus japonicus* [(1)]. We identified an infection-related gene from a *L. japonicus* mutant through genetic analysis of an EMS-induced mutant line impaired in infection thread formation. Based on phylogenetic analysis, syntheny and complementation assay, we confirmed that the identified gene is an orthologue of *ERF Required for Nodulation 1* (*ERN1),* encoding an AP2/ERF-type DNA-binding domain carrying transcriptional factor, previously identified in *Medicago truncatula* [(2,3)]. In contrast to *M. truncatula*, *L. japonicus* carries only one gene copy within the *ERN* genomic cluster. A detailed phenotypic characterization of two independent *ern1* mutant lines in *L. japonicus* revealed that *LjERN1* fully controls bacterial entry via infection thread initiation and progression. Interestingly, the phenotypic defect of *Ljern1* displays common features but also differences compared to that of the previously characterized *M. truncatula ern1* or *ern2* mutants [(2,4)]. Importantly, we discovered that the CCaMK/CYCLOPS complex regulates positively *ERN1* expression. The predicted CYCLOPS binding site on the *ERN1* promoter was confirmed by transactivation assays and DNA-protein interaction assays *in vitro*. Our data reveal a central node of the transcriptional network that controls bacterial entry into plant cells.

References:

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POSTER 3-6 /LIGHTNING TALK/

Policing the Gate: Can Pea Plants Stop Rhizobial Cheats from Entering?

Annet Westhoek[1,2], F. Rehling[3], P. S. Poole[1], L. A. Turnbull[1]

[1] *Department of Plant Sciences, University of Oxford, Oxford, United Kingdom*
[2] *Systems Biology Doctoral Training Centre, University of Oxford, United Kingdom*
[3] *Department of Ecology, Philipps University Marburg, Marburg, Germany*

Legumes form symbioses with nitrogen-fixing soil bacteria called rhizobia. An intricate signalling process allows rhizobia to infect plant roots and form nodules. Inside nodules, rhizobia fix atmospheric nitrogen into ammonia and provide it to the plant (1). Rhizobial strains vary widely in how much nitrogen they provide and this affects crop yields (2). Despite some evidence to the contrary, there have been recent claims that legumes exert 'partner choice' and selectively form symbioses with rhizobia that provide more nitrogen (3,4). We tested whether peas exert such partner choice.

As many traits influence the ability of rhizobia to form nodules, the only unbiased test of partner choice requires the use of strains that differ in their ability to fix nitrogen, but nothing else. We developed sets of wild-type nitrogen-fixing strains and their respective *nifH* mutant non-fixing strains. Strains were distinguished using chromosomal *gusA* and *celB* marker genes and were otherwise completely isogenic. Peas were inoculated with different ratios of fixing to non-fixing strains. We found that the percentage of nodules containing the fixing strain exactly reflected the percentage of the fixing strain in the inoculum. We therefore found no evidence for partner choice.

Our results demonstrate that pea plants cannot exercise partner choice. This emphasizes the essential role of plant sanctions for plant and rhizobial fitness. In sanctioning, plants allocate fewer resources to established nodules providing little nitrogen (5). Ongoing work will focus on how such sanctions affect crop yields and populations of effective and less effective rhizobia in the soil.

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POSTER 3-7 /LIGHTNING TALK/

Molecular Control of Receptor Mobility Shifts during Rhizobial Infection

T. F. Stratil, P. Liang, C. Popp, S., M. Marín, J. Folgmann, Thomas Ott

Institute of Genetics, Ludwig-Maximilians-Universität München, Planegg-Martinsried, Germany

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Intracellular colonization of plant cells by symbiotic bacteria is a critical step for the host that requires stringent surveillance circuits at the plasma membrane to keep exclusive control over the infection process. Accumulating evidence suggests that such perception and signal transduction complexes are pre-formed in membrane compartments such as mesoscale membrane domains (MDs). However, neither the existence of pathway-specific MDs nor their controlled assembly has been demonstrated. Here, we unravelled the sequential organization of membrane-resident signalling proteins that are indispensible for the intracellular infection of *Medicago truncatula* roots by symbiotic bacteria. We identified actin, the flotillin FLOT4, the remorin SYMREM1 and the entry receptor LYK3 as essential molecular building blocks that are required and sufficient for the assembly of an infection-related MD in vivo. In addition we unravelled the mechanism that leads to the lateral immobilization of the LYK3 receptor in this MD upon rhizobial infection.

POSTER 3-8

A Deep Study of the Role of Different Regulatory Genes in the Symbiotic Abilities of *Sinorhizobium fredii* HH103: Inactivation of *nodD2* or *nolR* Enables this Strain for Nodulation with *Lotus japonicus*

S. Acosta-Jurado[1], F. Pérez-Montaño[1], D. N. Rodríguez-Navarro[2], Y. Kawaharada[3], I. Jiménez-Guerrero[1], P. Navarro-Gómez[1], H. Jin[3], S. U. Andersen[3], F. J. Ollero[1], F. J. López-Baena[1], N. Sandal[3], J. Stougaard[3], J.E. Ruiz-Sainz[1], José M. Vinardell[1]

[1] *Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Sevilla, Spain*
[2] *IFAPA, Centro Las Torres-Tomejil. Alcalá del Río, Sevilla, Spain*
[3] *Department of Molecular Biology and Genetics, Centre for Carbohydrate Recognition and Signalling, Aarhus University, Aarhus, Denmark*

Sinorhizobium Fredii HH103 is a rhizobial species exhibiting an extremely broad host-range that includes legumes forming determinate (such as soybean) and indeterminate nodules (Margaret et al. 2011, Vinardell et al. 2015). However, this strain fails to nodulate the model plant *Lotus japonicus*, although it is able to infect *Lotus burttii* roots by crack entry and induce the formation of Fix+ nodules in this plant.

In this work, by combining RNAseq and qPCR analyses, we have determined the set of HH103 genes whose expression is affected upon treatment with flavonoids. Three groups of genes differentially expressed upon treatment with genistein were identified: i) genes controlled by *nod* boxes, ii) genes regulated by *tts* boxes, and iii) genes not preceded by a *nod* box or a *tts* box, revealing a complex regulatory network. Interestingly, we have found differentially expressed genes not previously studied in rhizobia, and many of them appear not to be related to Nod factors or to the symbiotic type 3 secretion system (T3SS).

In addition, we will provide a map of the HH103 flavonoid-induced complex regulatory network which involves at least four different transcriptional regulators: NodD1, NodD2, TtsI, and NolR. These studies are particularly interesting since we have recently found that inactivation of the regulatory genes NodD2 or NolR results in a change on the mode of infection of *Lotus burttii* (from crack entry to infection threads) and even enables HH103 to effectively nodulate *Lotus japonicus*.

This work was supported by projects P11-CVI-7500 and P11-CVI-7050 of the Junta de Andalucía, by projects AGL2012-38831 and BIO2011-30229-C01 of the Ministerio de Ciencia e Innovación of the Spanish government, and by the V Plan Propio of the University of Seville (VPPI-US).

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POSTER 3-9

Characterization of Rhizobial Strains Capable of Overcoming Restrictive Phenotypes of Pea (*Pisum sativum* L.)

Alexey Afonin[1], A. Sulima[1], V. Zhukov[1], I. Tichonovich[1,2]

[1] *All-Russia Research Institute for Agricultural Microbiology, Saint-Petersburg, Russian Federation*
[2] *Saint-Petersburg State University, Department of Genetics and Biotechnology, Saint-Petersburg, Russian Federation*

Garden pea (Pisum sativum L.) is capable of forming beneficial symbiosis with Rhizobium leguminosarum bv. Viceae, in the form of nitrogen fixing nodules. Different pea cultivars have a different specificity when choosing their microsymbiont. One of the most restrictive is the so-called "Afgan" phenotype. Only bacteria possessing the *nodX* gene responsible for acylation of the nod factor can overcome this restrictive phenotype. One of such strains is the strain A1, possessing a number of unique traits. A1 was able to form nodules with the RisNod4 non-nodulating mutants with a mutation in the receptor domain of sym37 receptor kinase. However, it was unable to form nodules with the K24 cultivar, possessing a mutation in the kinase domain of the sym37 gene. Computer modelling showed that the Risnod4 mutation renders the Sym37 receptor domain incapable of interacting with nod factors with and without acylation, regulated by the nodX, leading us to believe, that A1 strain possesses alternative signals to invoke nodulation.

Objective of this study was to characterize the genomic composition of A1 strain, compare it to strains incapable of nodulating RisNod4 plants and to isolate the genetic determinants responsible for increased symbiotic efficiency of the strain.

Three rhizobial strains were sequenced using the Illumina HiSeq2000 system: A1, TOM and 1026. Of them only A1 was able to suppress pea mutant phenotype. At least 90 putative genes were unique to A1 strain, but were closely related to proteins of other rhizobial species. A plasmid fragment, containing two genes homologous to srf genes of Mesorhisobium loti was found.

Strains, capable of nodulating peas with the "middle-eastern" phenotype or sym37 defective mutant plants were isolated from 25 nodules, 15 from middle-eastern and 10 from mutant plants. A particular strain was consistently capable of overcoming the K24 line mutation, leading us to suspect a nod-factor independent route of nodulation. Characterization of genomic composition of all the strains is underway.

This work was supported by Grant of President NSH-6759.2016.4 and RSF grant 14-24-00135.

POSTER 3-10

Colonization and Changes of Root Hair Morphologies Induced by *Rhizobium cellulosilyticum* on Different Legumes

Alexandra Díez-Méndez[1], Esther Menéndez[1], Paula García-Fraile[2], Raúl Rivas[1,3] and Pedro F. Mateos[1,3]

[1] *Departamento de Microbiología y Genética, Universidad de Salamanca, Spain*
[2] *Institute of Microbiology Academy of Sciences of the Czech Republic, Prague, Czech Republic*
[3] *Unidad Asociada de I+D Universidad de Salamanca (USAL-CSIC) (IRNASA), Spain*

Rhizobium cellulosilyticum ALA10B2^T is a rhizobial strain originally isolated from a peculiar environment, different from legume nodules: the decaying wood of a *Populus alba* tree. This strain was described as able to produce large amount of hydrolytic enzymes, probably involved in the breakdown of plant cell wall polymers (1). This strain was also tested for nodulation in *Macroptilium atropurpureum* and *Phaseolus vulgaris,* showing no nodule formation, and in *Medicago sativa* in which the formation of few ineffective nodules was described (1).

Therefore, in this study, we tested the strain behaviour during the first steps of the *R. cellulosilyticum* ALA10B2^T-*Medicago sativa* symbiotic interaction (15 dpi). We designed laboratory assays, in which we inoculated alfalfa plants with RFP-tagged *R. cellulosilyticum* ALA10B2^T. Our results showed that *R. cellulosilyticum* ALA10B2^T is a great root colonizer and induces root hair redirections with no damages on the root architecture, despite of its ability to produce a large amount of hydrolytic enzymes involved in the plant-cell degradation. On the other hand, *R. cellulosilyticum* ALA10B2^T we did not observe infection threads. When we co-inoculated *Ensifer meliloti* 1021, a nodulating strain of *Medicago sativa*, with the RFP-tagged *R. cellulosilyticum* ALA10B2^T, alfalfa plants presented a normal nodulation pattern, but this last strain was not observed inside any infection thread.

The same inoculation assays were carried out with RFP-tagged *R. cellulosilyticum* ALA10B2^T in other legumes (*Phaseolus vulgaris Medicago truncatula*, and *Trifolium repens),* obtaining equivalent results: great colonization of root surface without any damage of the root hairs cell wall, as well as root hairs re-directions and no infection threads.

Therefore, *R. cellulosilyticum* ALA10B2^T seems to be a good candidate to be used as model strain for deepening in the study of the molecular interactions between rhizobial hydrolytic enzymes and plants roots.

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POSTER 3-11

Comparative Analysis of Tubulin and Actin Cytoskeleton Organization in Symbiotic Nodules of Pea (*Pisum sativum* L.)

Anna B. Kitaeva[1], K. N. Demchenko[1,2], T. Timmers[3], V. E. Tsyganov[1]

[1] *Laboratory of Molecular and Cellular Biology, All-Russia Research Institute for Agricultural Microbiology, Saint-Petersburg, Russia*
[2] *Laboratory of Anatomy and Morphology, Komarov Botanical Institute, Saint-Petersburg, Russia*
[3] *Laboratory of Plant Microorganism Interactions (CNRS UMR2594, INRA UMR441), Castanet-Tolosan, France*

The pea symbiotic nodule is a useful model to study the molecular and cellular mechanisms of the legume-*Rhizobium* symbiosis. It is obvious that tubulin cytoskeleton rearrangements have a crucial role in nodule organogenesis and functioning. However, only a limited number of studies are available on the role and structure of the tubulin cytoskeleton during nodule development.

Using immunocytochemical analysis and confocal laser scanning microscopy, the three-dimensional microtubular organization of each nodule histological zone in pea nodules was analyzed and linked to the developmental processes during nodule cell differentiation. The pea wild-type SGE (1) and corresponding mutant lines SGEFix$^-$-1 (*sym40*), SGEFix$^-$-2 (*sym33*) (2) were used. The *Sym40* gene is orthologous to the *M. truncatula EFD* gene (3). The *Sym33* gene is orthologous to the *M. truncatula IPD3* gene (4).

This study has revealed the important role of endoplasmic microtubules and actin microfilaments in the growth of the infection thread, the formation of the infection droplet and bacterial release into the host cell cytoplasm as well as in the orientation of bacteroids. It was also observed that rhizobial infection triggers an alteration in the specific orientation of cortical microtubules, which is characteristic for adjacent cells that remain uninfected. The alteration in orientation of actin microfilaments after bacterial release was not observed. The other particularity in actin cytoskeleton was high dense network around nuclei in different types of nodule cells.

Thus, it seems that tubulin and actin cytoskeleton can function at different steps of nodule cell differentiation.

This work was supported by Russian Science Foundation (16-16-10035).

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POSTER 3-12

High-throughput Transposon Mutagenesis Screening of Pea Symbiont *Rhizobium leguminosarum* to Investigate Colonization of the Germinating Pea Spermosphere and Radicle

Benjamin J. Perry[1], Rachel M. Wheatley[2], Vinoy K. Ramachandran[2], Philip Poole[2], Christopher K. Yost[1]

[1] *Integrated Microbial Systems and Society Laboratory, University of Regina, Regina, Canada*
[2] *Rhizosphere Lab, Department of Plant Sciences, University of Oxford, Oxford, United Kingdom*

The spermosphere represents the immediate soil environment surrounding a germinating seed (1). The spermosphere receives inputs of plant derived exudates through two mechanisms: first, passively during imbibition of a dry plant seed; and second, actively by the emerging plant radicle and eventual primary root. The spermosphere is an often over-looked, but potentially important, developmental stage for the establishment of a plant-microbe symbiosis as it represents the first opportunity for soil microbes to perceive and colonize a newly developing plant root system. To identify the genetic basis of fitness for colonizing the pea spermosphere high-throughput transposon screens using *mariner* transposon insertion sequencing (INSeq) were performed using the pea symbiont *Rhizobium leguminosarum* bv. *viciae* 3841 (Rlv3841) in the early and late spermosphere (2-3). Additional INSeq screenings were performed on Rlv3841 inoculated onto emerging pea radicles grown on water agar medium, to identify genes involved in the colonization of emerging pea radicles.

Three independently generated Rlv3841 mutant pools were screened in three parallel INSeq studies consisting of 40 pea spermospheres, or pea radicles, each. Each pea spermosphere or radicle was inoculated with an average of $3.6x10^6$ Rlv3841 *mariner* transposon mutants, totaling $1.4x10^8$ mutants screened within each independent experiment. Insertion site sequencing, processing, and mapping of 84 million *mariner* insertion tags resulted in recovered transposon insertion densities ranging from 70.3% to 84.2% for all potential *mariner* insertions. Data analysis with Bayesian and non-parametric resampling methods identified a LysR-like transcriptional regulator that is putatively important in early colonization of the spermosphere. Candidate genes were also identified with putative roles for radicle and late spermosphere colonization. The devised experimental approach provides a new way to study the spermosphere, and refinement of the methodology will improve the use of INSeq to identify genes involved in Rlv3841 colonization of germinating host spermospheres.

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POSTER 3-13

Implications of *Rhizobium* Cellulase CelC2 Heterologous Expression in Cereal Root Colonization

Lorena Celador-Lera[1], Esther Menéndez[1], Pedro F. Mateos[1,2], Raúl Rivas[1,2]

[1] *Department of Microbiology and Genetics/CIALE, Universidad de Salamanca, Spain*
[2] *Associated I+D Unit USAL-CSIC (IRNASA), Spain*

Engineering nitrogen-fixing interaction between microbes and cereals is essential for reaching a sustainable food production (1). A proper colonization and a correct establishment of the symbiosis-signaling pathway are essential for bacterial infection and nodule organogenesis. Preliminary studies have shown that *Rhizobium* cellulase CelC2 is essential in primary symbiotic infection and its tightly regulated production is required for root hair and functional nodule development in its host. *celC*-overproducing derivative strains extensively degraded plant cell wall in the inner legume nodules (2). Moreover, cellulase CelC2 is involved in cellulose biosynthesis (3). On the other hand, several studies reported that *Rhizobium* is able to colonise non-legume roots as endophyte, such as maize and rice (4, 5). In this study, we isolated 58 endophytic strains from maize roots and identified them based on 16S rRNA gene sequencing. Five strains of the total isolated endophytes were identified as *Rhizobium* and several plant growth promotion tests were carried out. Our results showed that an isolate belonging to *Rhizobium leucaenae* had the best performance in the conditions tested. This isolate also revealed cellulolytic activity and capability to synthesise exo-polysaccharides. In order to enhance this strain competitiveness for maize roots, we engineered a derivative strain expressing heterologously *R. leguminosarum* bv trifolii ANU843 cellulase CelC2. *R. leucaenae celC*$^{+}$ derivative strain had an increased cellulolytic activity under *in vitro* conditions respect to wild-type strain and also, is affected in its exo-polysaccharide production. Colonization assays using GFP-tagged *celC*$^{+}$ derivative revealed reduced its root colonization in comparison with wild-type inoculated plants. Furthermore, maize roots inoculated with *celC*$^{+}$ derivative exhibited an increment in root hair redirections with respect to wild-type inoculated and uninoculated plants. Interestingly, maize plants showed no significant differences in shoot and root length in the first steps of development (10 dpi) between wild-type and celC^{+} derivative. Moreover, root architecture remains intact under the tested conditions. These results suggest that the heterologous expression of *R. leguminosarum* bv trifolii ANU843 cellulase CelC2 in *R. leucanae* has no negative effects in maize root cell walls in the tested conditions.

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POSTER 3-14

Rhizobium sp Actively Colonizes Spinach (*Spinacia oleracea* L.) Roots

Alejandro Jiménez-Gómez[1], José David Flores-Félix[1], Paula García-Fraile[1], Pedro F. Mateos[1,3], Esther Menéndez[1] and Raúl Rivas[1,3]

[1] *Department of Microbiology and Genetics/CIALE, University of Salamanca, Salamanca, Spain*
[2] *Institute of Microbiology, Academy of Sciences of Czech Republic, Praha, Czech Republic*
[3] *Associated I+D Unit. USAL-CSIC (IRNASA), Salamanca, Spain*

Colonization is one of the most important process in which an interaction between bacteria and plant is involved. Its correct establishment determines the effectiveness of the association. Several studies showed that rhizobial strains form a successful colonization on root surfaces as a first step of the interaction (1). However, one of the strategies to ensure an effective microbial root attachment is the formation of biofilms. Considering that biofilm formation protects root-attached bacteria from desiccation, UV radiation and nutrient release (1-2), the search for bacterial strains, which are able to form these structures, become important for a proper establishment of bacterial-plant relationship.

In this study, we analyzed the ability of PEPV40, a strain isolated from *Phaseolus vulgaris* nodules and identified as *Rhizobium* sp., to form *in vitro* biofilms and to colonize spinach (*Spinacia oleracea* L.) roots, which is one of the most demanded leafy crops and harbors a high interest for human diets.

Our results showed that this strain effectively colonises and adheres to abiotic and biotic surfaces. GFP-tagged PEPV40 inoculation on spinach roots was observed daily by fluorescence microscopy, showing a gradually increased attachment to root surface and root hairs. Interestingly, this strain causes structural and morphologicchanges in root hairs, such as deformations and redirections at the tip during the initial period of plant development.

Moreover, we evaluated *in vitro* plant development after inoculation of spinach seedlings with the strain of this study, showing significant differences in shoot and root length with respect to uninoculated plants.

Our results showed that strain PEPV40 has the ability to effectively colonise spinach root surfaces, suggesting that its interaction with this spinach roots would be beneficial and being a potential candidate as inoculant for this crop.

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POSTER 3-15

Role of Cell-to-cell Communication during the Establishment of the Nitrogen-fixing Symbiosis

Martina Beck, L. Frances, F. Regad, A. Niebel, D. G. Barker, F. de Carvalho-Niebel

Laboratory of Plant Microbe Interactions (LIPM), INRA, CNRS, Université de Toulouse, Castanet-Tolosan, France

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The establishment of the nitrogen-fixing endosymbiotic interaction between *Medicago truncatula and Sinorhizobium meliloti* involves major and specific host cell reprogramming as the infecting microbe progresses across outer root cell layers to reach the inner cortical nodule primordium. Accumulating evidence suggests that direct cell-to-cell communication plays a central role in this highly specialized transcellular infection process which involves the formation of successive apoplastic "infection thread" compartments. Unique cell wall channels in plants, so-called plasmodesmata (PDs), are essential for direct cell-to-cell communication and we are therefore studying PD distribution and symplastic fluxes during early symbiotic infection stages by using *in vivo* markers and specific staining protocols. Furthermore, we have identified several genes coding for PD-associated proteins that are upregulated in the *Medicago* nodule infection zone. *In silico* analysis of additional *Medicago* transcriptomic data sets has identified candidate genes encoding β-1,3-glucanases, PD-localized proteins or PD-callose binding proteins, which are related to *Arabidopsis* homologues previously involved in the regulation of PD flux during root development and biotic interactions. We show that two of them, coding for β-1,3-glucanase hydrolytic enzymes are induced in rhizobial inoculated roots and results of their subcellular localization, tissue-specific expression profiles and functional studies during infection will be presented and discussed.

POSTER 3-16

Symbiotic Genes Regulated by ERN1/ERN ERF Transcription Factors

Maria Fernanda Guerrero-Molina[1], L. Frances[1], M. R. Cerri[2], A. Breakspear[3], M. Parniske[1], A. Niebel[1], J. Murray[3], F. de Carvalho-Niebel[1]

[1] *Laboratory of Plant Microbe Interactions (LIPM), INRA, CNRS, Université de Toulouse, Castanet-Tolosan, France*
[2] *Faculty of Biology, University of Munich (LMU), Institute of Genetics, Martinsried, Germany*
[3] *John Innes Centre, Norwich, United Kingdom*

Legumes improve their mineral nutrition through nitrogen-fixing root nodule symbioses with soil rhizobia. Bacterial infection of legumes is regulated by a number of transcription factors, including the *Medicago truncatula* ERF-type transcription factors *ERN1 (ERF Required for Nodulation 1)* and its close homolog *ERN2,* which shows partially overlapping expression patterns (1-3). Recent phenotypic analysis of mutant lines revealed an extreme symbiotic phenotype for the double *ern1 ern2* mutant line compared to either single mutants (5), demonstrating functional redundancy between them during early stages of infection. Accordingly, the *ENOD11* and *ENOD12* target genes have their NF-elicted expression abolished in the double mutant background while the expression of other symbiotic genes was unaffected (5). To elucidate whether *ERN1* and *ERN2* regulate the same set of genes during rhizobial infection we analyzed the expression by q-RT-PCR of a number of infection-related symbiotic genes (4) in roots of single and double mutant lines at different times points after rhizobial inoculation. These analyses revealed new symbiotic genes whose expression is dependent on both ERN TFs. Additional transcriptomic strategies aimed at identifying symbiotic genes regulated by both ERN1/ERN2 transcription are currently being designed and will be presented.

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POSTER 3-17

The Pleiotropic Phenotype of a *Bradyrhizobium Diazoefficiens* Δ*ecfG* Mutant under Free-living and Symbiotic Conditions

Raphael Ledermann, Barbara Müller, Janine Wülser, Jean-Malo Couzigou, Anja Michel, Julia A. Vorholt, Hans-Martin Fischer

ETH Zürich, Institute of Microbiology, Zürich, Switzerland

The general stress response (GSR) in α-proteobacteria regulates cellular responses that result in the decrease and/or repair of stress-induced damage. Typically, the GSR confers cross-protection by integrating a variety of different stress signals (1). Core elements of the response involve the extracytoplasmic function σ-factor EcfG, its cognate anti-σ-factor NepR and the anti-anti-σ-factor PhyR. Previously it was shown that *Bradyrhizobium diazoefficiens* (formerly known as *B. japonicum*) Δ*ecfG* or Δ*phyR* mutants are more susceptible to heat shock and desiccation compared to the wild type, and the symbiotic interaction of these mutants with soybean and mungbean is impaired (2). More recently, we have performed a detailed phenotypic characterization of the *B. diazoefficiens* Δ*ecfG* mutant both under free-living and symbiotic conditions. We found that the symbiotic defect of the mutant is first manifested by the delayed, restricted formation of infection threads and nodule primordia of which many arrest at an early stage. Careful titration of various stress conditions for free-living bacteria revealed that Δ*ecfG* and Δ*phyR* mutants not only are more susceptible to high temperature and desiccation but also to alkaline pH, salts, osmolytes, a chemical oxidant, UV radiation, and organic solvents. By contrast, no difference between the mutants and the wild type was observed regarding sensitivity against detergents, various heavy metals, and acidic pH. To monitor induction and activity of the GSR under different stress conditions, we have established an EcfG-dependent reporter system using β-galactosidase and fluorescent proteins as readout. Also, mutant strains lacking putative components of the upstream signaling cascade to PhyR-NepR-EcfG were constructed and phenotypically characterized under symbiotic and stress conditions. Preliminary results indicate substantial functional redundancy in the sensing segment of the GSR network. Accordingly, mutant strains lacking multiple candidate sensory components are currently being constructed using a novel markerless deletion mutagenesis protocol (3).

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POSTER 3-18

Transcripto-proteomic Dissection if Differentiated Bacteroid Physiology using *Bradyrhizobium* Strains in Interaction with Soybean and *Aeschynomene* Legume Hosts

Florian Lamouche[1], Anaïs Chaumeret[1], Samuel Mondy[1], Attila Szücs[2], Thierry Balliau[3], Michel Zivy[3], Eric Giraud[4], Eva Kondorosi[1,2], Attila Kereszt[2], Peter Mergaert[1], Benoit Alunni[1]

[1] *Institute for Integrative Biology of the Cell, UMR9198 CEA/CNRS/Université Paris Saclay, Gif-sur-Yvette, France*
[2] *Institute of Biochemistry, Biological Research Centre HAS, Szeged, Hungary*
[3] *UMR de Génétique Végétale, PAPPSO, Ferme du Moulon, Gif-sur-Yvette, France*
[4] *Laboratoire de Symbioses Tropicales et Méditerranéennes, UMR IRD/SupAgro/INRA/UM2/CIRAD, Montpellier, France*

To circumvent the paucity of nitrogen sources in the soil Legume plants evolved the ability to interact with nitrogen-fixing soil bacteria referred to as rhizobia. In the frame of this symbiotic interaction, legumes form root organs called nodules, where bacteria are housed intracellularly and become bacteroids. Depending on the host plant, bacteroids can remain similar to cultured bacteria (unmodified morphotype, like in soybean nodules) or can undergo cell enlargement coupled to endoreduplication (elongated and spherical morphotypes, like in *Aeschynomene afraspera* and *Aeschynomene indica* nodules respectively). Why such bacteroid diversity exists is still unclear. Nevertheless, Oono and Denison showed that differentiated bacteroids may be more efficient for nitrogen fixation. To get insights on bacteroid differentiation and its consequences on the bacterial physiology, we used two closely-related *Bradyrhizobium* strains (*Bradyrhizobium diazoefficiens* USDA110 and *Bradyrhizobium* sp. ORS285) able to nodulate these three plants two by two, resulting in the production of all three types of bacteroids. We thus compared the transcriptome (RNA-seq) and the proteome (LC-MS/MS) of bradyrhizobia in interaction with soybean and *Aeschynomene* plants and compared it to the free-living condition. Integration of the data provides a set of candidate bacterial functions for further functional analysis.

POSTER 3-19

Web-based Visualization of Expression Data and Gene Co-expression Networks in *Lotus japonicus*

Terry Mun, Asger Bachmann, Simon Kelly, Jens Stougaard and Stig U. Andersen

Centre for Carbohydrate Recognition and Signalling, Aarhus University, Denmark

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The model legume *Lotus japonicus* is able to participate in a wide range of host-microbe interaction along the symbiont-pathogen spectrum. Nitrogen-fixing legume-rhizobia symbiosis has been extensively investigated (1), while unpublished data has shown that well-studied phytopathogens, *Ralstonia* (2) and *Verticillium* (3), are capable of colonizing and infecting *L. japonicus*. Despite progress in the studies of the interactions *Lotus* can partake in, tools used to visualize, interpret and analyze so called "big data" in a user-friendly manner is lacking within the research community. Here we describe a toolkit, named Expression Atlas (ExpAt), developed as part of the *Lotus* Base project (Mun *et al.* in preparation), that allows users to visualize gene co-expression patterns conveniently and quickly in a modern, standards-compliant web browser, by leveraging on recent advances in browser architecture and adoption of HTML5.

The *Lotus* Base ExpAt database currently includes data from the LjGEA project (4), with in-house mapping of *Lotus* probes against v3.0 of the annotated genome; early transcriptomic changes of: *Lotus* root hairs to symbionts and purified compounds involved in symbiosis; and *Lotus* roots to symbiotic and pathogenic fungal exudates (5). It is possible to perform hierarchical agglomerative clustering with various linkage methods and metrics, in order to cluster a generated heatmap. In addition, we have created the Correlation Network tool (CORNET), which allows users to generate gene co-expression networks and overlay these with custom lists of genes of interest. We demonstrate the potential of the tool by superimposing gene expression induction by Nod factor treatment in root hairs on a co-expression network generated based on non-cell-type-specific data.

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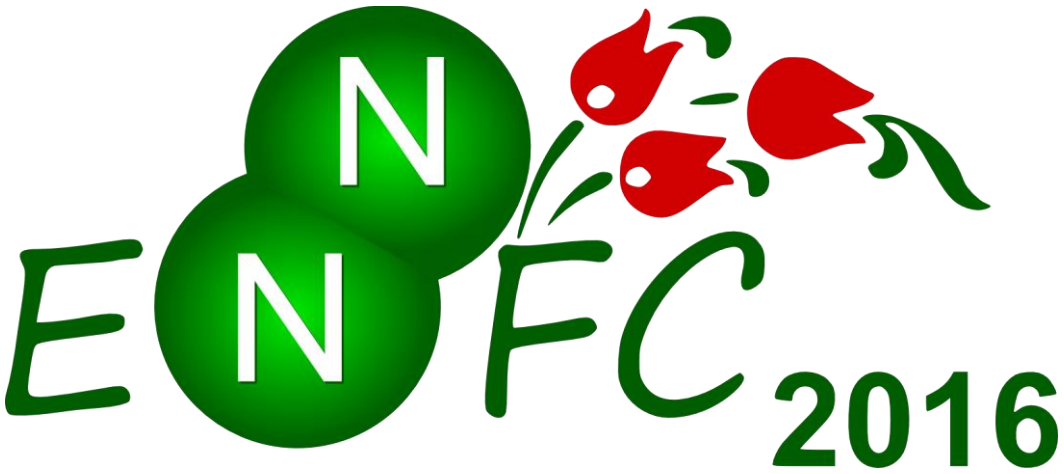

POSTER SESSION 4

Interplay of Nitrogen-fixing and Mycorrhizal Symbioses

Room Orion

POSTER 4-1 /LIGHTNING TALK/

Do You Want to Join the Complex? Towards the Identification of New CCaMK/CYCLOPS Interactors

A. Sandré, C. Cathebras, P. Bellon, Rosa Elena Andrade Aguirre, X. Gong, M. Parniske

Faculty of Biology, Genetics, University of Munich (LMU), Martinsried, Germany

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During evolution plants recruited genes previously involved in arbuscular mycorrhiza (AM) symbiosis to establish a new interaction with nitrogen-fixing bacteria called root nodule (RN) symbiosis (1). The establishment of these symbioses is triggered by a signaling transduction pathway featuring perinuclear calcium oscillations, conceptually deciphered by CCaMK (1, 2). CCaMK phosphorylates CYCLOPS (2, 3) and forms a protein complex (CCaMK/CYCLOPS), that transcriptionally activates downstream genes required for specific developmental responses in AM and RN symbiosis (3, 4). Additionally, we found that this complex is involved in regulating lateral root formation in *Lotus japonicus*.

Although this complex has a strategic place upstream different pathways, little is known about how those are differentially induced. Moreover, rice orthologs of CCaMK and CYCLOPS are able to restore nodulation of corresponding legume mutants (2, 5), indicating that the acquisition of the ability to induce the transcriptional network required for root nodule symbiosis does not rely on functional adaptation of CCaMK or CYCLOPS.

Recent results indicate that the CCaMK/CYCLOPS interaction appears to be the core of a larger dynamic complex. For example, DELLA is recruited into the CCaMK/CYCLOPS complex to activate transcription of *RAM1* during AM (4). It is likely that additional components of the complex may help to determine the correct transcriptional responses appropriate for different symbiotic and developmental processes. The CCaMK/CYCLOPS interactome and the mechanisms by which novel interactors contribute to specific transcriptional responses required for the two distinct symbioses and lateral root development are under investigation.

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POSTER 4-2 /LIGHTNING TALK/

The Process of Bacteroid Differentiation in Pea (*Pisum sativum* L.) is Controlled by Symbiotic Genes that Regulate the Expression of the NCR Gene Family

Igor A. Tikhonovich[1,2], Marina S. Kliukova[1,2], Olga A. Kulaeva[1], Alexander I. Zhernakov[1], Vladimir A. Zhukov[1]

[1] *All-Russia Research Institute for Agricultural Microbiology, St.-Petersburg, Russia*
[2] *Saint-Petersburg State University, St.-Petersburg, Russia*

The ability of leguminous plants to control the symbiotic bacteria in nodules is based on their capability to trigger the differentiation the bacteria into the symbiotic form - bacteroids. In Legumes of IRLC clade, this differentiation is promoted and mediated by short plant peptides named NCR (nodule-specific cysteine-rich) peptides (1, 2). NCR peptides are highly divergent in amino acid composition, so each species possesses its own spectrum of NCR peptides. The gene family composed of more than 600 members encoding NCR peptides has recently been characterized in *Medicago truncatula* (2, 3). In pea, only some members of this gene family are known (characterized as early nodulins) (4).

The development of the "next generation sequencing" (NGS) methods along with the availability of the unique pea mutants made it possible to describe the gene family encoding NCR peptides in pea and to study the genetic control over the NCR gene family expression. Using both the publically available and original data on pea nodule transcriptome sequencing (5), we found more than 200 nodule-specific transcripts encoding peptides that belong to NCR family. The transcripts were classified into groups according to expression level in 12-, 21- and 28-days old wild type nodules (based on original RNAseq data). Then, also using RNAseq, their expression was evaluated in nodules of several symbiotic mutant lines with defects in nodule development and functioning. Virtually all NCR genes were downregulated in *sym31* and *sym33* mutants (forming ineffective nodules with no signs of bacteroid differentiation). Only "late" NCR genes were downregulated in *sym40* and *sym42* mutants (forming nodules with abnormal morphological differentiation of bacteroids), whereas the "early" NCR genes were either upregulated or unchanged. Almost only "early" NCR genes were significantly upregulated in *sym26* and *sym27* mutants (forming nodules with prematurely degraded symbiotic structures). The expression level of ten selected NCR genes was evaluated by real time PCR on early stages of nodule development, confirming different regulation of expression for different groups of NCR genes in pea.

The fact that mutant phenotype is associated with mis-expression of only some NCR genes points at possible key role of the particular NCR peptides in regulation of bacteroid differentiation in pea. In general, the different expression of NCR genes in mutants with different degree of bacteroid differentiation allows us to outline the scheme of the plant control over the microsymbiont's fate during nodule development.

This work was supported by the Russian Science Foundation [grant number 14-24-00135].

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POSTER 4-3 /LIGHTNING TALK/

Expression of a Rhizobial Efflux System and its Associated Transcriptional Regulator during Nodule Development

A. Welker, S. Krysenko, D. Mitić, M. Göttfert and Anna Zdyb

Institute of Genetics, Technische Universität Dresden, Dresden
E-mail: Anna_Maria.Zdyb@tu-dresden.de

Symbiotic nitrogen-fixing bacteria are exposed to various toxic plant compounds during the establishment of a successful symbiosis. Bacteria are equipped with several efflux systems that allow the extrusion of harmful chemicals encountered in their environment. Rhizobial genome sequences reveal the presence of several efflux systems belonging to different families. In the alfalfa symbiont *Sinorhizobium meliloti* strain 1021, 14 efflux systems have been identified (1). In transcriptome analyses, the genes *SMc03167* and *SMc03168* – the deduced proteins are similar to the multi-drug resistance proteins EmrB and EmrA of *E. coli*, respectively – were reported to be inducible by luteolin, a plant signal known to induce nodulation genes (2). Using a transcriptional *emrA-gusA* fusion, we demonstrated that the gene is inducible by several flavonoids also by quercetin, which is not an inducer of nodulation genes. This suggests that the gene is not regulated directly by NodD, which is the activator of nodulation genes. Upstream of *emrA*, a TetR-type regulator (EmrR) is encoded. EmrR binds to palindrome-like sequences within the *emrA-emrR* intergenic region (3). Our investigation revealed that *emrR* is also inducible by apigenin. After integration of the *emrR-lacZ* fusion into an *emrR* mutant background, the fusion was no longer inducible by apigenin. However, the expression level in the non-induced strain was significantly higher than in the wild-type background. This suggests that EmrR acts as a repressor, which regulates the transcription of *emrAB* and of its own gene. Interestingly, a mutation of *emrR* but not of *emrA*, impaired symbiosis with alfalfa (3, 4 and unpublished results). This might indicate that a proper regulation of *emrAB* is essential for the interaction of *S. meliloti* with alfalfa. To address this issue, we used reporter gene fusions of *emrA* and *emrR* and studied their expression in nodules of alfalfa and *Medicago truncatula*. Preliminary results indicate that EmrA is expressed in the infection zone of alfalfa and *M. truncatula* nodules. Expression of EmrR was detected in the infection zone and also throughout the fixation zone. There was no expression detected in the infection threads of mature nodules neither during the initial infection thread formation. This result suggests that the expression of this efflux system is only triggered at a specific point of nodule development.

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POSTER 4-4 /LIGHTNING TALK/

Rhizobial Competition: Getting to the Root of the Problem

Marcela A. Mendoza-Suárez, B. A. Geddes, C. Sánchez-Cañizares, P. S. Poole

Department of Plant Sciences, University of Oxford, Oxford, United Kingdom

The symbiosis formed between plants of the legume family with nitrogen-fixing bacteria called rhizobia is of major global importance in agricultural systems [1]. As free-living cells in the soil, competition amongst them is crucial, as the most competitive rhizobial strain will be the one able to infect the legume host and form a nodule. Unfortunately, competitiveness and effectiveness, which is the ability of rhizobia to reduce N_2 to ammonia and transfer it to the plant, are independent rhizobial traits. Therefore, legumes tend to become nodulated by highly competitive but not necessarily effective rhizobial strains, resulting in a less optimal plant growth [2]. This is a major problem for nutrition in countries where beans, which are particularly likely to infection by strains of varying effectiveness, are an important protein source. Research focused on the identification of both competitive and highly efficient nitrogen-fixing strains has been limited because so far, the only way to screen rhizobial strains has been to isolate individual strains and compare them one at a time in large-scale plant growth assays.

The principal aim of this project is to engineer tools to quickly assess competitive and effective rhizobial strains in a large population of native soil rhizobia allowing us to compare multiple strains at once and thus to carry out a high-throughput screening for competitive and efficient strains.

We have developed novel reporter plasmids, which include the biomarkers *gusA* or *celB* under the control of a synthetic *nifH* promoter. These reporter plasmids were constructed by an integrated high-throughput strategy using the latest 'Golden Gate' cloning technique [3].

We transferred these plasmids to *Rhizobium leguminosarum* bv. viciae *3841* and followed the competition methodology from Sánchez-Cañizares & Palacios (2013). The competition assays carried out with our reporter plasmids show that they do not affect the competitiveness of *Rhizobium leguminosarum* bv. viciae 3841. Thus, our results demonstrate that by using synthetic biology we can produce a suitable bioreporter that will allow us to carry out a high-throughput screening for competitive and efficient strains. In order to achieve this, the next step in this project will be the addition of barcodes to enable rapid identification of successful strains, allowing us to compare multiple strains at once.

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POSTER 4-5 /LIGHTNING TALK/

Plant as an Evolutionary Driver of Symbiotic Microbiome

Anna Igolkina, O. Onishchuk, O. Kurchak, E. Chirak, E. Andronov, N. Provorov

ARRIAM, St-Petersburg, Russia

The main hypothesis of the current project is the strong plant influence on soil microbiome structure. Testing it we applied the modern molecular methods and multilevel bioinformatic analysis. Our project was aimed to detect plant-microbe parallel evolution in nitrogen-fixing *Rhizobium*-legume symbiotic system, to be specific, between its two components: rhizobial symbiotic gene *nodA* and plant receptor gene *nfr5*. These genes play an essential role in symbiotic partners recognition.

Preparing the initial data we massively sampled three wild growing (from one compact territory) legume plants (*Vicia, Lathyrus, Trifolium*): plant tissue, root nodules and corresponding rhizosphere soil samples. Finally, for each particular plant we created three DNA pools: from plant, nodules and soil. We used degenerate primers to construct amplicon libraries. These libraries were analyzed with pyrosequencing (*nodA*) and Sanger sequencing (*nfr5*).

Using high-throughput data we developed the original computational pipeline for detection of plant-driven variability in soil microbiome. It consisted of several hierarchical steps from phylogenetic analysis to parallel diversity analysis of both bacterial and plant genes. We first performed OTU-picking for total *nodA* gene sequences and detected the frequency-dependent selection during bacterial transition into a host plant. Next, the diversity analysis on haplotype and nucleotide levels showed the increased diversity of *nodA* alleles in root nodule population compared to the soil one. Haplotype frequencies shift in root-nodule population reveals symbiosis-specific selection patterns how a plant chooses specific bacteria.

On the next step we studied the source of the variability in root nodule population and developed the modification of dn/ds statistics adapted to our data. We found high level of both synonymous and nonsynonymous substitutions in root-nodule population and significantly increased value of dn/ds statistics. These results characterise the type of natural selection acting on the root-nodule population and reveal positions in nodA gene under this selection.

Finally, we compared nucleotide diversity values between *nfr5* gene in plants and *nodA* genein root-nodule populations and found these values linearly dependent.

All obtained results demonstrated quite clear and spectacular picture of co-evolutionary forces in symbiotic systems and especially diversity analysis showed the guide role of a plant in evolution of soil microbiome.

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POSTER 4-6 /LIGHTNING TALK/

Effect of Phosphate Solubilization on Nitrogen Fixation in Clover

Kritarth Seth[1], Derrick Moot[1], Maureen O'Callaghan[2], Hayley Ridgway[1]

[1] *Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln, New Zealand*
[2] *AgResearch Ltd, Lincoln Research Centre, Christchurch, New Zealand*

The ability of legumes to fix nitrogen (biological nitrogen fixation; BNF) in symbiosis with rhizobia is limited by soil P availability. This is because BNF requires 5 - 10% more energy than assimilation of the same amount of soil N (1). Thus, the balance between N and P is critical for effective BNF.

Phosphorus, being highly reactive, is the most immobile element in soil with only 5 to 30% of fertilizer inputs utilized by plants. The remainder complexes with metal ions and accumulates in soil as insoluble, unusable deposits. Microbial phosphate solubilization (PS) via the secretion of organics acids has been reported to make more P available for plant uptake. Many bacterial species can solubilise P *in vitro* but most have failed to do so *in planta*. This means there is no large scale bacterial commercial product for PS. The objective of this study was to screen nodule inhabitants of clover for PS and to study the effects of higher P availability on BNF.

A total of 2220 nodule inhabiting bacteria were collected from four sites with contrasting long term P fertilization histories, using white (*Trifolium repens*) and subterranean (*T. subterraneum*) clovers as bait plants. Isolates were screened for their ability to solubilise P *in vitro* using media containing highly insoluble hydroxyapatite (HA) as the sole source of P. Only 79 (3.6%) of bacteria were found to solubilise P. More isolates originating from subterranean than white clover nodules could solubilise P. This suggests an active host recruitment ($p<0.05$) of phosphate solubilizing bacteria (PSB). A higher ($p<0.05$) proportion of PSB was also recovered from plants grown in soil that had received high levels of P, suggesting adaptation of bacteria to solubilise P in these soils.

Only 25 of the 79 PSB were *Rhizobium spp.*, despite all originating from within clover nodules. Of these, 11 isolates (*R. leguminosarum* bv. *trifolii*) were able to nodulate white clover and fix nitrogen. When white clover plants were supplemented with minimum nitrogen and HA as the only P source, all 11 isolates increased plant growth in comparison with the uninoculated control. When used to inoculate white clover, the best isolates produced a 73% increase in dry weight compared with our standard commercial inoculant *R. leguminosarum* bv. *trifolii* TA1.

The best isolates found in this study increased plant growth by a combined effect from fixing N and solubilising P. These dual function inoculants provide the potential for sustainable P utilization and improved nitrogen fixation.

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POSTER 4-7 /LIGHTNING TALK/

Quorum Sensing Controls Phenotypic Heterogeneous Expression of the Autoinducer Synthase Gene *trai* via Copy Number Control of pNGR234*a* in the Plant Symbiont *S. Fredii* NGR234

Katrin Petersen[1], J. Grote[1], D. Krysciak[1], C. Schmeisser[1], K. U. Förstner[2], H. Krishnan[3], W. R. Streit[1]

[1] *Biozentrum Klein Flottbek, Abteilung für Mikrobiologie und Biotechnologie, Universität Hamburg, Hamburg, Germany*
[2] *Core Unit Systems Medicine, Universität Würzburg, Würzburg, Germany*
[3] *Plant Genetics Research Unit, United States Department of Agriculture-Agricultural Research Service, University of Missouri, Columbia, Missouri, United States*

Sinorhizobium Fredii NGR234 is a plant symbiont which is able to form nitrogen-fixing nodules in over 120 plant genera. Its genome encodes for two autoinducer (AI) systems in which the TraI system is localized on the symbiotic plasmid pNGR234*a* that is in part highly similar to the *A. tumefaciens* Ti plasmid.

qPCR analyses of copy numbers of pNGR234a of single AI deletion mutants *S. fredii* NGR234 Δ*traI* and Δ*ngrI* and the corresponding double AI-synthase mutant indicate the partial or complete lack of AI molecules affects the copy number of the pNGR234*a* replicon. In general, the copy number was altered in response to mutations affecting the AI regulons and by the addition of external AI molecules. The increased copy number observed in the absence of any AI basically eliminated the previously described phenotypic heterogeneous expression of the *traI* gene4 and caused a low level expression of virtually all genes on pNGR234*a*. RNA-seq data in the background of a Δ*traI*, a ⊿*ngrI* and a Δ*traI*⊿*ngrI* double mutant indicate that the copy number control of pNGR234*a* and the phenotypic heterogeneity are linked with to two novel ORFs identified on the symbiotic replicon. The two ORFs encode for a 51 aa and a 143 aa protein located in the region of the oriV of pNGR234*a*. We have designated these ORFs *repX* and *repA0* and both have previously not been reported. Both proteins are unique to broad host range rhizobia and not present in *A. tumefaciens* or related bacteria affiliated with the rhizobiales. Overexpression of *repA0 in NGR234 wt cells* increases the copy number of pNGR234*a*. Therefore we speculate that both ORFs are part of NGR's unique tool box that enables it to nodulate such a wide spectrum of legume plants.

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POSTER 4-8

Characterisation of Symbiotic and Non-symbiotic Rhizobial Diversity in an Agricultural Soil

Amalia Soenens[1], Santiago de la Peña[1], Juan Imperial[1,2]

[1] *Universidad Politécnica de Madrid. Centro de Biotecnología y Genómica de Plantas, Pozuelo de Alarcón, Madrid, Spain*
[2] *Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain*

Rhizobia are a diverse group of soil α- and β-proteobacteria able to establish endosymbioses with plants belonging to the Fabaceae (legumes) group. In this symbiosis the legume plants are able to feed from the nitrogen fixed by the rhizobia, which makes them independent from any external fixed nitrogen supply. Common rhizobia belong to the genera *Rhizobium*, *Sinorhizobium (Ensifer), Neorhizobium, Mesorhizobium* and *Bradyrhizobium*. It is noteworthy that both endophytic and exophytic rhizobia have been detected in many of the non-legume plant microbiome projects carried out lately (1,2,3,4,5). This suggests that rhizobia could be universally present in the plant microbiomes. For symbiotic rhizobia isolation, "plant traps" have traditionally been used. In order to also characterise the non-symbiotic rhizobia, we have developed a strategy consisting of a combination of semi-selective media already described, with the use of specific genus markers developed in our laboratory.

Our objective is to develop an adequate strategy to allow us to isolate and characterise the symbiotic and non-symbiotic rhizobia populations in a given soil. This soil will then be used as the source of rhizobia where different plants, legumes and non-legumes will be grown in order to determine their effect on these populations and to uncover the roles rhizobia play in the biology of those plants.

We describe the characterisation of *R. leguminosarum, Sinorhizobium (Ensifer)* sp., *Neorhizobium* sp., and *Bradyrhizobium* sp., symbiotic and non-symbiotic populations of an agricultural soil where no legumes have been previously grown. We find a general paucity of symbiotic rhizobia: *R. leguminosarum* and *Sinorhizobium (Ensifer)* sp., numbers are low and no *Bradyrhizobium* sp. strains have been isolated. The observed high abundance of non-symbiotic *Neorhizobium* sp. strains isolated suggest an important role of these rhizobia in this soil.

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POSTER 4-9

Competition of α- and β-rhizobia for Legume Infection

M. Lardi, A. Gandolfi, G. Purtschert, S. Bolzan de Campos, L. Eberl and Gabriella Pessi

Department of Plant and Microbial Biology, UZH Zurich, Switzerland
gabriella.pessi@botinst.uzh.ch

Recently, β-proteobacteria of the genera *Cupriavidus* and *Burkholderia* have been shown to be able of establishing nitrogen-fixing symbiosis with legumes (the so called β-rhizobia). Previous studies reported that some legumes can be nodulated by both α- and β-rhizobia and suggested the existence of genetic and physiological factors that determine the preference of certain legumes for α- or β-rhizobia. *Mimosa* spp. have been shown to have a clear preference for β- rhizobia.

In this study, we investigated the abilities of six β-rhizobial strains to compete for nodulation of different legumes such as *Mimosa pudica*, *Vigna unguiculata*, *Phaseolus vulgaris* and *Macroptilium atropurpureum*. While *Burkholderia phymatum* was found to be the most competitive strain on three out of the four tested legumes (*M. pudica*, *V. unguiculata* and *P. vulgaris*), *B. tuberum* was the most successful strain on *M. atropurpureum*. Moreover, *B. phymatum* and *B. mimosarum* seem to be able to co-exist inside *P. vulgaris* and *M. pudica* nodules. We are currently performing *in vitro* competition tests using differentially tagged β-rhizobial strains (DsRed and GFP) as well as competition experiments between β- and α-rhizobial strains for legumes infection.

POSTER 4-10

Response of the Endophytes Plant-growth Promoters *Enterobacter* sp. UYSO10 and *Shinella* sp. UYSO24 to Sugarcane Roots Exudates

Cecilia Taulé[1], A. Lima[2], R. Duran[2] and F. Battistoni[1]

[1] *Departamento de Bioquímica y Genómica Microbianas (BIOGEM), Instituto de Investigaciones Biológicas Clemente Estable (IIBCE), MEC, Montevideo, Uruguay*
[2] *Unidad de Bioquímica y Proteómica Analíticas, Mixed unit: IIBCE-Instituto Pasteur de Montevideo, Montevideo, Uruguay*

Plant growth-promoting bacteria can be used to improve the sustainability of crops by reducing the fertilization applied. *Enterobacter* sp. UYSO10 and *Shinella* sp. UYSO24 were isolated from surface sterilized stems of Uruguayan sugarcane cultivars. Greenhouse experiments showed that both strains are plant growth promoters of sugarcane plants (cv. LCP85384) (Taulé et al 2012, 2016). Moreover, the colonization and infection process of sugarcane plants were determined, and they were defined as endophytes (Taulé et al 2016).

The aim of this work was to study the response of strains UYSO10 and UYSO24 to the sugarcane cv. LCP85384 exudates. In this sense, a whole proteomic approach was conducted applying the *Difference In Gel Electrophoresis* (DIGE) technique. For that, first roots exudates were collected from cv. LCP85384 micropropagated plants, after 5 days of growth in modified Murashing and Skog (MS) liquid medium. After that, bacterial strains were incubated (48 h) in modified MS medium, in presence or absence of roots exudates, and total proteins were extracted. Samples proteins were labeled as DIGE technique required and the electrophoresis were carried on in 24 cm gels. The gels were scanned and the images analyzed using DeCyder Differential Analysis Software. Results showed that strain UYSO10 expressed 34 differential proteins while UYSO24 29 proteins. Differential expressed proteins were excised from gel and identified by MALDI-TOF-TOF. Results of the peptide identification will be presented.

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POSTER 4-11

Role of TTSS on Symbiotic Establishment between Non-Photosynthetic Bradyrhizobia and Leguminous Plants

Pongdet Piromyou[1], Pongpan Songwattana[1], Watcharin Yuttavanichakul[1], Kamonluck Teamtisong[1], Panlada Tittabutr[1], Michael Göttfert[2], Eric Giraud[3], Neung Teaumroong[1]

[1] *School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand*
[2] *Technische Universität Dresden, Institut für Genetik, Dresden, Germany*
[3] *IRD, Laboratoire des Symbioses Tropicales et Méditerranéennes, UMR IRD/SupAgro/INRA/UM2/CIRAD, Campus International de Baillarguet, TA A-82/J, Montpellier, France*

Type 3 effector proteins secreted via the bacterial type III secretion system (T3SS) are not only the virulence factors of pathogenic bacteria, but also influence on symbiotic relationships between rhizobia and leguminous plants. On the basis of bradyrhizobia 16s rRNA gene sequences similarity, the phylogenetic tree could be divided into two major clusters. The member in the cluster 1 belonged to various groups of *Bradyrhizobium* species. The strains STM6978, DOA9 and SUTN9-2 were also grouped into this cluster. The *B. elkanii* clade was clearly separated from PB and Non-PB groups. In case of T3SS (*rhcJ*) gene, the strains STM6978, DOA9 and SUTN9-2 slightly separated from bradyrhizobial group. Genomic comparison of bradyrhizobial strains SUTN9-2, DOA9 and STM6978 revealed a gene cluster encoding a T3SS that is similar to those found in rhizobia. In addition, the genes which encode the structural core components of the T3SS, the cluster contains several open reading frames that are specific to *B. elkanii* USDA61. However, several ORFs within the cluster (upstream region) are not conserved in all tested strains. On the basis of phylogenetic tree data and gene arrangement, it seems that the T3SS of *A.americana* isolated strains (SUTN9-2 and DOA9) and USDA61 may share the same origin. In contrast, these data could also raise another hypothesis that STM6978, SUTN9-2 and DOA9 acquired T3SS from *B. elkanii* USDA61. Then, the bradyrhizobial strains STM6978, SUTN9-2 and DOA9 have been evolved their T3SS separated from USDA61. For functional analyses, the *rhcJ* genes of strains SUTN9-2, DOA9 and STM6978 were disrupted. These mutations had a cultivar-specific effect on nodulation properties. The T3SS of DOA9 and STM6978 displayed the negative effect on *Vigna radiata* cv. SUT4 and *V. radiata* cv. KPSII nodulation. In contrast, the T3SS of SUTN9-2 showed the positive effect on nodulation of most tested legumes.

POSTER 4-12

The Influence of Rhizobia Strains on the Yield Formation of Broad Beans (*Vicia Faba*) in the Different Soil Types

Laila Dubova, Alise Šenberga, Ina Alsiņa, Liene Strauta

Institute of Soil and Plant sciences, Latvia University of Agriculture, Jelgava, Latvia

Broad beans are healthy source of nutrition for people. Moreover they are valuable crop in different crop rotation systems. The effective symbiosis between the plant and soil microorganisms has significant influence on legume productivity. The efficiency of symbiosis depends on the biological and environmental factors, including soil type. The experiment was established to evaluate the effectiveness of *Rhizobium leguminosarum* strains on legumes growth and productivity in the different soil types. *Rhizobium sp.* strains (RP023, RP003, RV407 and RV505) were obtained from the Rhizobium Collection of Latvia University of Agriculture. In the field experiments broad bean (*Vicia faba* L. var. major Harz.) cultivars 'Bartek' and 'Karmazyn' were grown in sandy, loamy sand and peat soil. Seeds were treated with suspension of bacteria before sowing. Control variant was without microorganism treatment. Plant length, weight of dry matter, weight and formation of nodules were analyzed at the beginning of flowering stage (BBCH 60-61). Number of pods, seed yield and protein content in seeds were analyzed at the end of experiment. Experiments were done in 4 replications during vegetation period of Year 2014 and 2015. Data were examined by Analysis of Variance, using Student criteria and Correlation analysis between plant growth parameters and protein content in seeds. Results showed that the growth and yield responses of both bean cultivars to inoculation with *Rhizobium sp.* strains vary in the different soil types. There were no significant differences among variants of plant fresh weight and dry weight. Inoculation with *Rhizobia* promoted the increase of seed weight. *Rhizobia* treatment increased the protein accumulation in the broad bean seeds but degree of effectiveness ranges between *Rhizobia* strains. Therefore, is crucial to find suitable *Rhizobia* strain or combination of strains for different soil types.

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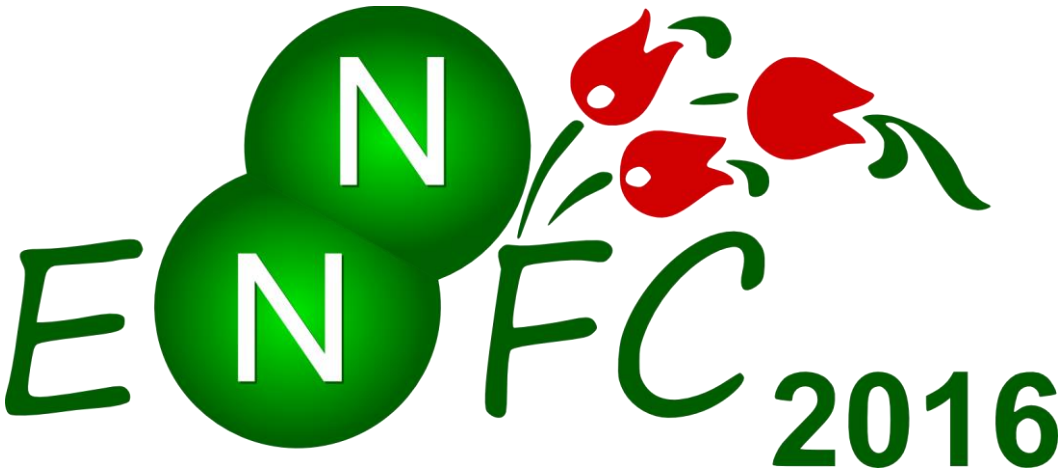

POSTER SESSION 5A

Functioning of the N-fixing Symbioses /Bacteria/

Room Mercure

POSTER 5A-1 /LIGHTNING TALK/

sRNA-mediated Regulation of the Cell Cycle Master Regulator CtrA in *Sinorhizobium meliloti*

Marta Robledo[1,2], J. P. Schlüter[1], L. O. Loehr[1], U. Linne[3], S. Albaum[4], J. I. Jiménez-Zurdo[2], A. Becker[1]

[1] *LOEWE Center for Synthetic Microbiology and Faculty of Biology, Philipps-Universität Marburg, Germany*
[2] *Grupo de Ecología Genética de la Rizosfera, Estación Experimental del Zaidín (CSIC), Granada, Spain*
[3] *Department of Chemistry, Philipps-Universität Marburg, Germany*
[4] *Center for Biotechnology CeBiTec, Universität Bielefeld, Germany*

Small untranslated RNAs (sRNAs) are widespread post-transcriptional regulators that modulate fundamental aspects of bacterial physiology in response to environmental conditions. Regulation of the cell cycle to retard chromosome replication, segregation and/or cell division under stress conditions is crucial for bacteria. In α-proteobacteria, tight control of the cell cycle is also necessary to achieve genome replication only-once per generation, as observed in *Caulobacter crescentus,* or to accomplish cellular differentiation within legume host root cells, like most symbiotic α-rhizobia. Coordination of DNA replication and cytokinesis in model α-proteobacteria is ensured by cyclically changing concentrations of the conserved transcriptional regulators DnaA, GcrA and CtrA[1]. DnaA and GcrA control transcription of genes involved in early cell cycle events, whereas the essential master regulator CtrA directly controls genes primarily involved in late cell cycle events, motility and asymmetry[2]. We have recently reported a conserved sRNA modulating expression of *dnaA* and *gcrA* under stress conditions in α-rhizobia[3]. However, post-transcriptional mechanisms controlling cell division are still poorly explored.

The *S. meliloti* trans-encoded sRNA GspR (Growth Stop Phenotype RNA) was selected from a phenotypic screening of sRNA overexpression strains. The growth arrest phenotype was observed in all *Sinorhizobium* species carrying GspR homologs. Northern blot analysis confirmed production of this sRNA under different growth and stress conditions. Its predicted secondary structure contains three stem-loops (SL). CopraRNA-based predictions of mRNA targets of SL1 and SL3 showed an enrichment for cell division-related genes. Transcriptome and proteome analyses identified several genes which were differentially expressed dependent on GspR. Both approaches identified *ctrA*, which was less abundant in the *gspR* overexpression strain, along with proteins linked to cell motility and cellular metabolism. GspR-dependent altered translation of *ctrA* mediated by mRNA base-pairing with SL1 was experimentally confirmed by an eGFP-based reporter system and compensatory changes in sRNA and mRNA.

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POSTER 5A-2 /LIGHTNING TALK/

Inactivation of PhaR Involved in Poly-beta-hydroxybutyrate Accumulation in *Bradyrhizobium japonicum* USDA110 and its Pleiotropic Effects

Shogo Nishihata[1], Kosei Tanaka[2], Shinji Takanaka[1,2] and Ken-ichi Yoshida[2, 3]

[1] *Department of Agrobioscience, Kobe University, Kobe, Japan*
[2] *Organization of Advanced Science and Technology, Kobe University, Kobe, Japan*
[3] *Department of Science, Technology and Innovation, Kobe University, Kobe, Japan*

Bradyrhizobium japonicum USDA110 accumulates poly-beta-hydroxybutyrate (PHB) in the cells grown with an excess of carbon sources. Previously we demonstrated that PhaP proteins (phasins) stabilizing PHB granules played important roles in PHB accumulation in USDA110 (1). PhaR repressor was suggested to regulate expression of the *phaP* genes but remained to be elucidated experimentally. In this study, we created and analyzed a *pha*R-deficient mutant strain to investigate its changes in various phenotypes, including bacterial growth, PHB accumulation, and symbiotic nitrogen fixation. In addition, PhaR was produced and purified in *Escherichia coli* to analyze its DNA binding in vitro.

When the *pha*R-deficient mutant was cultured in non-PHB-accumulating conditions, among the genes involved in PHB accumulation, transcription levels of *phaP1*, *phaP4*, and *phbZ1* were elevated significantly, indicating that PhaR could be involved in transcriptional repression of the two phasins, PhaP1 and PhaP4, as well as the PHB degrading enzyme, PhbZ1. Indeed, in vitro experiments, including gel mobility shift and DNase I foot print analyses, revealed that PhaR specifically bound to the promoter regions of these genes, identifying its function as transcriptional repressor.

When cultured in PHB-accumulating conditions, growth of *pha*R-deficient mutant was impaired, and its intracellular PHB accumulation was reduced more pronouncedly than in the *phaP4*-deficient mutant. On the other hand, it secreted twice more exopolysaccharides (EPS) than both the parental and the *phaP4*-deficient strains. The results suggest that elevated expression of the two phasins and/or the PHB degrading enzyme might cause the less efficient PHB accumulation. Moreover, inactivation of *pha*R might switch the cellular metabolism from PHB accumulation to EPS secretion, and thus caused changes in the metabolism might restrict the cell growth. Unexpectedly, the *pha*R-deficient mutant showed somehow elevated resistance to thermal stresses, although non-PHB-accumulating mutants of other microorganisms reportedly exhibited less tolerance to various stresses.

When soybean was infected with *pha*R-deficient mutant, nodules were formed almost similarly as infected with the parental strain, and normal growth of soybean plant was observed without supplemental nitrogen sources. The results suggest that neither the less efficient PHB accumulation nor the enhanced EPS secretion might affect the establishment of symbiosis and nitrogen fixation. Since PHB may serve as energy storage compound in bacteroides, we are currently investigating effects of the *pha*R inactivation on efficiency in bacterial outgrowth from matured nodules.

References:

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POSTER 5A-3 /LIGHTNING TALK/

Insertion Sequencing in *Rhizobium leguminosarum* bv. viciae 3841

Rachel M. Wheatley, Vinoy K. Ramachandran, Philip S. Poole

Department of Plant Sciences, University of Oxford, Oxford, United Kingdom

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Rhizobium leguminosarum bv. *viciae 3841* belongs to the symbiovar *viciae* that establishes nitrogen-fixing symbiosis with *Viciae* legumes such as agriculturally important pea (*Pisum sativum*) and vetch (*Vicia craccca*). Microarray transcriptomic and bioinformatics studies carried out in *Rhizobium* have suggested many gene functions but they do not necessarily identify those important for growth in a particular environment or those required for symbiosis (1-2). Insertion sequencing (INSeq) can be used to study gene fitness at the genome scale. It involves subjecting large libraries of Mariner transposon insertion mutants to high-throughput sequencing to assess how mutants are altered in growth and survival (3).

InSeq has been coupled with a four-state Hidden Markov Model (HMM) for analysis (4) which classifies genes as growth-essential, -defective, neutral or -advantaged.

INSeq has been used to investigate a variety of biological questions in Rlv3841; including pea root attachment, growth in the pea rhizosphere, growth on a C4 carboxylic acid carbon source, and for growth at low oxygen tensions. The HMM was able to successfully assign gene phenotype classifications to 7316 genes. Our INSeq genetic screen of Rlv3841 grown on glucose and succinate at both 21% and 1% [O_2] enabled the identification of novel transcriptional regulators needed for growth on different carbon sources and oxygen levels.

Growth on succinate also required a significant proportion of cell envelope genes, suggesting restructuring of the cell surface, involving the PrsD-PrsE type I secretion system and EPS production. We carried out an INSeq genetic screen with Rlv3841 inoculated onto pea seedlings and allowed to grow for 5 days in the rhizosphere. Three different mutant libraries were analysed: the input mutants, the mutants retrieved from the rhizosphere, and the root attached mutants. A total of 64-million transposon insertion reads were sequenced, and an insertion density of 88% of all potential mariner insertion sites in the input library was obtained. Subsequent analysis has allowed identification of the genes required for growth specifically in the rhizosphere and genes required specifically for root attachment over 5 days colonisation.

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POSTER 5A-4 /LIGHTNING TALK/

MucR is Required for Transcriptional Activation of Conserved Ion Transporters to Support Nitrogen Fixation of *Sinorhizobium fredii* in Soybean Nodules

Jian Jiao[1,2,3], Li Juan Wu[1,2,3], Biliang Zhang[1], Yue Hu[1,2,3], Yan Li[1,2,3], Xing Xing Zhang[1,2,3], Hui Juan Guo[1,2,3], Li Xue Liu[1,2,3], Wen Xin Chen[1,2,3], Ziding Zhang[1] and Chang Fu Tian[1,2,3]

[1] *State Key Laboratory of Agrobiotechnology, and College of Biological Sciences, China Agricultural University, Beijing, China*
[2] *Key Laboratory of Soil Microbiology, Ministry of Agriculture, China Agricultural University, Beijing, China*
[3] *Rhizobium Research Center, China Agricultural University, Beijing, China*

To achieve effective symbiosis with legume, rhizobia should fine-tune their background regulation network in addition to activating key genes involved in nodulation (*nod*) and nitrogen fixation (*nif*). Here, we report that an ancestral zinc finger regulator, MucR1, other than its paralog, MucR2, carrying a frameshift mutation, is essential for supporting nitrogen fixation of *Sinorhizobium Fredii* CCBAU45436 within soybean nodules. In contrast to the chromosomal *mucR1*, *mucR2* is located on symbiosis plasmid, indicating its horizontal transfer potential. A MucR2 homolog lacking the frameshift mutation, such as the one from *S. fredii* NGR234, can complement phenotypic defects of the *mucR1* mutant of CCBAU45436. RNA-seq analysis revealed that the MucR1 regulon of CCBAU45436 within nodules exhibits significant difference compared with that of free-living cells. MucR1 is required for active expression of transporters for phosphate, zinc, and elements essential for nitrogenase activity (iron, molybdenum, and sulfur) in nodules but is dispensable for transcription of key genes (*nif/fix*) involved in nitrogen fixation. Further reverse genetics suggests that *S. fredii* uses high-affinity transporters to meet the demand for zinc and phosphate within nodules. These findings, together with the horizontal transfer potential of the *mucR* homolog, imply an intriguing evolutionary role of this ancestral regulator in supporting nitrogen fixation.

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POSTER 5A-5 /LIGHTNING TALK/

Stringent Response-mediated Transcriptional Changes in the *Medicago-Sinorhizobium* Root Nodule Symbiosis

Kathrin Wippel and Sharon R. Long

Department of Biology, Stanford University, Stanford, CA, USA

During the interaction with its host plant *Medicago sativa* (alfalfa), *Sinorhizobium meliloti* encounters several stages of environmental and nutritional changes, which require metabolic and physiological adjustment. Global gene expression in bacteria is altered upon nutrient stress via the stringent response. In general, when sensing uncharged tRNA molecule, the ribosome-bound protein RelA synthesizes the alarmone ppGpp. ppGpp, as well as the transcriptional regulator DksA bind RNA polymerase (RNAP) and shift the lifetime of certain RNAP/promoter complexes, thereby leading to down- and upregulation of specific gene sets.

We have previously shown that both *relA* and *dksA* are important in free-living conditions of *S. meliloti* as well as in symbiosis with alfalfa (1,2). The *relA* mutant phenotypes are very severe: *relA* is indispensible for growth on minimal medium and for nodulation on alfalfa. Detailed transcriptomic analysis on a *relA* mutant strain upon carbon or nitrogen starvation was reported earlier (3). To understand which gene expression changes mediated by the stringent response are necessary and sufficient for the establishment of symbiosis, we performed comparative global transcription profiling via our customized Affymetrix Symbiosis Chip (4) on bacterial wild type, Δ*relA*, and a previously isolated *relA* suppressor strain (5). Cells were grown in culture and induced by the plant flavonoid luteolin. Comparing *relA* to wild type, we found differential regulation of genes for exopolysaccharide biosynthesis, motility, glycine betaine synthesis, and structural proteins. However, no typical symbiosis genes were affected in the mutant; in contrast, *nodE* and *nodG*, as well as the ECF sigma factor gene *rpoE9* were slightly upregulated. There were more transcriptional changes between *relA* and its suppressor than between *relA* and the wild type, suggesting that the suppressor might even overshoot some gene regulation changes. In addition, there are distinct differences between wild type and suppressor, suggesting a lack or addition of specific regulations.

The suppressors initially identified (5) induce nitrogen fixation-deficient nodules on alfalfa, whereas newly isolated suppressors are fix+. All of these suppressors are point mutations in the RNAP β or β' subunits (RpoB or RpoC). We are defining the transcriptomes of three-week old nodules (bacteroids and plant tissue) from the different suppressor types, wild type, and a Δ*relA* strain overexpressing *dksA* (which results in wild type-like behavior), to decipher transcript requirements specific for nodulation and fixation in the context of stringent response. In a second approach, we are assessing the question of which gene transcripts are necessary for symbiosis to occur under ppGpp-deficient conditions by comparing wild type nodules to Δ*relA* mutant-induced nodules on *M. truncatula*, taking advantage of the fact that Δ*relA* nodulates *M. truncatula* in a wild type-like manner.

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POSTER 5A-6

A Regulatory Protein Encoded by *rosR* Affects Protein Secretion and Envelope Integrity of *Rhizobium leguminosarum* bv. *trifolii*

Monika Janczarek[1], Kamila Rachwał[1], Magdalena Kopycińska[1], Aleksandra Boguszewska[2], Marek Tchórzewski[2]

[1] *Department of Genetics and Microbiology, Institute of Microbiology and Biotechnology, Maria Curie-Skłodowska University, Lublin, Poland*
[2] *Department of Molecular Biology, Institute of Microbiology and Biotechnology, Maria Curie-Skłodowska University, Lublin, Poland*

Rhizobium leguminosarum bv. *trifolii* is capable of establishing a symbiotic relationship with *Trifolium pratense*. Previously, *rosR* encoding a global regulatory protein which positively influences EPS synthesis and plays a significant role in early stages of the symbiosis was identified and characterized in this bacterium (1). RosR is a C_2H_2-type zinc-finger protein belonging to the family of rhizobial Ros/MucR transcriptional regulators. Transcriptome profiling of a strain carrying a *rosR* mutation revealed the role of RosR in the synthesis of cell-surface components, motility, and other cellular processes (2).

In this work, we studied the influence of the mutation in *rosR* on profiles of extracellular, membrane and periplasmic proteins in *R. leguminosarum* bv. *trifolii* and identified proteins which significantly differed quantitatively or qualitatively between the *rosR* mutant and the wild-type strain. We established that the Rt2472*rosR* mutant secreted significantly higher amounts of an autoaggregation protein (RapA1), a cadherin-like protein, a RTX-like toxin, and flagellar components than the wild-type Rt24.2. Also, several differences were observed in the membrane protein profiles of Rt2472 and Rt24.2. Among the proteins whose levels were significantly higher in the *rosR* mutant in relation to the wild-type strain were those identified as phasin-like proteins, a flagellin, and a component of the ABC-type transport system. By contrast, a few other proteins were importantly less abundant in the *rosR* mutant in comparison to the wild-type profile. Among them were an outer membrane protein YaeT, an ATP synthase β-subunit, and a DNA-binding protein with a periplasmic domain binding a membrane lipoprotein. In the case of periplasmic protein fractions of Rt2472 and Rt24.2, the most conspicuous differences between these strains concerned two proteins the amounts of which were decreased in the *rosR* mutant profile in relation to that of the wild-type: a protein identified as a catalase/peroxidase HPI and a hypothetical protein. In addition, alterations in the topography and cell surface properties of these bacteria were found using atomic force microscopy (AFM).

This work was supported by a grant of the National Science Centre no 2012/07/B/NZ1/00099.

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POSTER 5A-7

A *Rhizobium leguminosarum* bv viciae DNA Region Involved in Host-specific Symbiotic Efficiency

Alejandro Sanz, Lu Jiangkun, David Durán, Laura Rubio-Sanz, Jose Palacios

Centro de Biotecnología y Genómica de Plantas (CBGP), and E.T.S.I. Agrónomos, Universidad Politécnica de Madrid. Campus de Montegancedo, Pozuelo de Alarcón, Madrid, Spain

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The symbiotic nitrogen fixation in legume nodules is the final outcome of a complex interaction between plant and rhizobia. In this interaction, multiple compounds produced either by the plant macrosymbiont (flavonoids, lectins, different kinds of peptides, etc) of by the bacterial microsymbiont (Nod factors, EPS, LPS, etc) participate in a sophisticate molecular dialogue that governs the specificity of the interaction (1). In spite of the number of signals involved, the degree of specificity of the symbiosis is highly variable. In some cases, several legumes can be nodulated by the same rhizobial strain. In the case of *Rhizobium leguminosarum* bv. viciae, plant species belonging to four different genera (*Pisum*, *Lens*, *Lathyrus*, and *Vicia*) are permissive for the establishment of efficient nitrogen fixation in nodules. The basis for the ability of this bacterium to interact with different legumes is not fully understood at molecular level.

Analysis of a set of randomly generated mutants of *R. leguminosarum* bv viciae UPM1137 allowed the identification of a mutant impaired on its symbiotic association with pea, inducing only white, ineffective nodules with this host. Interestingly, the mutant retains the wild-type ability to effectively nodulate lentil plants, a phenotype that we designate as Host-specific symbiotic efficiency (Hse). Additionally, the mutant is impaired in the growth in rich media such as TY. Complementation of the mutant with a *R. leguminosarum* bv. viciae gene bank has allowed the identification of a 3-kb DNA region able to restore the Hse phenotype. The role of the genes present in the *hse* region is currently being analysed, and the results of this analysis will be presented in the communication.

References:

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POSTER 5A-8

Characterisation of Quorum Sensing Proficient Pigeon Pea Nodulating Rhizobia for their Symbiotic Competence and Plant Growth Promotion

Jitendrapuri Gosai, G. Archana

Department of Microbiology and Biotechnology Centre, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India

Pigeon pea (*Cajanus cajan*) is cultivated on about 4.79 M ha soil in 22 countries. The legume is extremely rich in nitrogen with about 25% protein in dried seeds and is mainly nodulated by *Rhizobium*, *Sinorhizobium* and *Bradyrhizobium* spp. Many rhizobia have been reported to employ multiple quorum sensing (QS) systems regulating collective set of genes as a function of cell density and including many phenotypes governing the symbiotic potential. The QS systems of pigeon pea rhizobia have however not been hitherto characterized. Since the QS systems generally comprise of one or more hierarchically arranged sets of luxI-luxR homologues and an array of autoinducer-N-acyl homoserine lactone (AHL) molecules, we studied the circuitries and the types of AHLs produced by pigeon pea nodulating strains.

Nodule occupants of pigeonpea were isolated and identified by 16S rDNA sequencing to be *Rhizobium* or *Sinorhizobium* spp. Their autoinducer profiles were studied using thin layer chromatography and high performance liquid chromatography. Our results indicated the presence of short length AHLs including C6- and 3-O-C8-HSL as well as long chain length AHL molecules. Presence of *sinRI* and *traRI* circuits and *expR* orphan receptor was detected using polymerase chain reaction. There were variations in both the types of AHL molecules as well in the distribution of the gene circuits among the five isolates tested. The nodule isolates were found to promote the growth of the host plant significantly when bioinoculated as compared to the plants without any inoculants. Since rhizobia, apart from biological nitrogen fixation, have also been reported to have other plant growth promoting (PGP) properties such as auxin production, we monitored these in the strains. Swarming motility, exopolysachharide production and biofilm formation ability are some of the phenotypes reported to be under the control of QS and directly influence the symbiotic competence. Chemical inhibition of the quorum sensing resulted in an increase in the biofilm formation and inhibition of swarming motility in some isolates indicating the role of QS in the promotion of symbiotically important phenotypes (SIP). Quantitative real time PCR of genes regulating SIPs upon AHL stimulation will be reported.

POSTER 5A-9

Comparison of *Rhizobium leguminosarum* Determinate and Indeterminate Nodules on Legumes by RNA-Seq Analysis

Robert T. Green[1], Alison K. East[1,2], R. Karunakaran[1], J. Allan Downie[1], Philip S. Poole[1,2]

[1] *Department of Molecular Microbiology, John Innes Centre, Norwich, United Kingdom*
[2] *Department of Plant Sciences, University of Oxford, United Kingdom*

Strains of *Rhizobium leguminosarum* biovar *phaseoli* form nitrogen-fixing nodules on *Phaseolus vulgaris* (common bean). The ability to nodulate a particular plant is specified by the symbiosis (Sym) plasmid. Previously the Sym plasmid (pRP2) of *R. leguminosarum* bv *phaseoli* strain 4292 (Rlp4292) was replaced by the pea/vetch nodulation-specific Sym plasmid, pRL1JI, to make *R. leguminosarum* bv *viciae* strain A34 (RlvA34) (1). With the exception of the symbiosis plasmid, these two stains have an identical genome composed of a chromosome and three plasmids. Their Sym plasmids confer the ability to nodulate either bean or pea plants, which form intrinsically different nodule types. In bean, determinate nodules are formed which do not maintain an active meristem and undifferentiated bacteria can be cultured from mature nodules. Galegoid legumes, such as pea (*Pisum sativum*), form indeterminate nodules where the bacteria are terminally differentiated, showing altered cell shape and a highly endoreduplicated genome (2).

As bacterial strains Rlp4292 and RlvA34 share such a large common genome but nodulate different plants, this makes them extremely useful in investigating differences between determinate and indeterminate nodules. We undertook RNA-Seq analysis of Rlp4292 and RlvA34 isolated from both free-living cultures, and mature bean and pea nodules, respectively. Analysis of of genes differentially expressed in both Rlp4292 and RlvA34 bacteroids compared to free-living cells revealed functions that are common to both determinate and indeterminate mature nodules. Differential gene expression in nodules from either bean or pea, reveals how determinate and indeterminate nodules differ from each other. Results presented show how gene expression reflects conditions within nodules and the functions of bacteroids in the two different nodule types.

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POSTER 5A-10

Evolution of N_2-fixing Symbiosis through Acquisition of Tripartite Mobile Genetic Elements

Tim L. Haskett[1], J. J. Terpolilli[1], A. Asrat[1], G. O'Hara[1], W. Reeve[1], P. Wang[1], J. T. Sullivan[2], C. W. Ronson[2] and J. P. Ramsay[3]

[1] *Centre for Rhizobium Studies, Murdoch University, Western Australia, Australia*
[2] *Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand*
[3] *CHIRI Biosciences Research Precinct, Faculty of Health Sciences, Curtin University, Bentley, Australia*

Bacterial integrative & conjugative elements (ICEs) are chromosomally-integrated DNA islands that excise to form circular molecules capable of self-transmission via conjugation. Lateral transfer of a subordinate ICE group, termed symbiosis-islands, converts non-symbiotic Mesorhizobia into nitrogen-fixing plant symbionts in a single evolutionary step. Here we document the discovery of a novel "tripartite" ICE-form, which exist as three separate chromosomally-integrated DNA segments that recombine and horizontally transfer as a single circular element. Genome sequence comparisons of *Mesorhizobium ciceri* strain WSM1271 with environmental and laboratory-isolated symbiosis-island exconjugants revealed that three distinct DNA regions were transferred from WSM1271 during conjugation. Using mutagenesis, quantitative PCR and artificial "mini-ICE" elements, we demonstrated that the tripartite ICE, ICE*Mc*Sym^{1271}, encoded three site-specific recombinases that catalysed recombination between three distinct pairs of DNA attachment sites (*att*) located at each ICE*Mc*Sym^{1271}-chromosome junction. The position and orientation of each *att* site was such that the sequential action of each recombinase in any order was predicted to resolve ICE*Mc*Sym^{1271} into a single circular ICE. Quantitative PCR demonstrated that recombination of each of three *att*-site pairs was coordinated and was additionally co-stimulated by the quorum-sensing regulator TraR. Distinct tripartite ICEs were identified in various *Biserrula* and *Lotus*-nodulating species, and horizontal transfer of these tripartite ICEs was also demonstrated. In each case, transfer conferred on the recipient an ability to form a specific symbiotic relationship with the legume species associated with the donor Mesorhizobium, however, the effectiveness of symbiotic N_2-fixation was largely dependent on the chromosomal background. These discoveries highlight the great diversity, peculiarity and plasticity of mobile genetic elements and clearly illustrates that we have only begun to scratch the surface in understanding the role which horizontal transfer of symbiosis genes plays in the evolution of symbiotic N_2-fixing bacteria.

POSTER 5A-11

Functional Analysis of the Two-component Regulatory System hFixL-FxkR in *Sinorhizobium meliloti*

Lourdes Girard[1], Chouhra Talbi[1], Alma Reyes-González[1], Patricia Rivera[1], Susana Rodríguez[1] and David Zamorano-Sánchez[2]

[1] *Programa de Dinámica Genómica, Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México*
[2] *Department of Microbiology and Environmental Toxicology, University of California, Santa Cruz, United States*

Transcriptional control of the *fixK* gene in *Rhizobium etli* and *Rhizobium leguminosarum* bv. viciae is governed by a two component signal transduction system that diverts from the conventional FixL-FixJ cascade that occurs in model rhizobia: a) the *fixJ* gene is absent; b) besides the sequence motifs found in other FixL proteins, RetFixL and RlegFixL proteins possess a nonheme-binding N-terminal PAS domain and also a C-terminal FixJ-like receiver domain present in response regulators; c) a novel response regulator that belongs to the OmpR/PhoB superfamily (*fxk*R dubbed *fixK* regulator) is strictly necessary to activate *fixK*f expression in response to low oxygen concentration. FxkR and hFixL related proteins are shared with other bacteria that belong to the rhizobial group (1).

In *Sinorhizobium meliloti*, nitrogen fixation is regulated in response to oxygen concentration through the FixL-FixJ two-component system (TCS). Besides this conserved TCS, the field isolate SM11 also encodes the hFixL-FxkR TCS, which is responsible for the microoxic response in *Rhizobium etli*. Through genetic and physiological assays, we evaluated the role of the hFixL-FxkR TCS in *S. meliloti* SM11. Our results revealed that this regulatory system activates the expression of a *fixK*f orthologue (*fixK*a), in response to low oxygen concentration. Null mutations in either hFixL or FxkR promote upregulation of *fixK*1, a direct target of FixJ. Furthermore, the absence of this TCS translates into higher nitrogen fixation values as well as higher expression of *fixN*1 in nodules. Individual mutations in each of the *fixK*-like regulators encoded in the *S. meliloti* SM11 genome do not completely abolish *fixN*1 or *fixN*2 expression, pointing towards redundancy among these regulators. Both copies of *fixN* are necessary to achieve optimal levels of nitrogen fixation. This work provides evidence that the hFixL-FxkR TCS is activated in response to low oxygen concentration in *S. meliloti* SM11 and that it negatively regulates the expression of *fixK*1, *fixN*1 and nitrogen fixation (2).

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POSTER 5A-12

Host-range Determinants of the Divergent Nod-containing *Bradyrhizobium* Strain DOA9

Pongpan Songwattana[1], Rujirek Noisangiam [2], Kamonluck Teamtisong[3], Panlada Tittabutr[1], Nantakorn Boonkerd[1], Eric Giraud[4], Neung Teaumroong[1*].

[1] *Institute of Agricultural Technology, School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima, Thailand*
[2] *National Bureau of Agricultural Commodity and Food Standards, Ministry of Agriculture and Cooperatives, Ladyao Chatuchak Bangkok, Thailand*
[3] *The Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima, Thailand*
[4] *Laboratoire des Symbioses Tropicales et Méditerannéennes (LSTM,) TA A-82/J, Montpellier, France*

The first symbiotic megaplasmid was elucidated in *Bradyrhizobium* sp. DOA9 which originally isolated from the root nodules of the *Aeschynomene Americana* from Thailand. The highly divergent nodulation (*nod*) genes were harbored and broaden the host-range for nodulation on the root of most plants from the Dalbergioid, Millettioid, and Robinioid tribes. DOA9 mutants carrying the loss-of-function nod-factor biosynthesis (Δ*nodB* and Δ*nodA2*) can completely abolished the nodulation in all plant tests. Interestingly, the *nodA*1 mutant (Δ*nodA1*) displayed a wild-type number of nodules in Aeschynomene plants (*A. americana* and *A. afraspera*). However, Δ*nodA1* was significantly affected to nodulation in other plant tests. These results suggesting that DOA9 required nod-factors for nodule formation. The additional and divergent *nodA* genes (divergent *nodA1* and *nodA2*) likely expand the diversity of Nod-factor acyl chains, and might broaden the host range of the DOA9. This hypothesis was confirmed by complementation of *nodA1*, a wild-type number of nodule events were restored in siratro inoculation. The T3SS mutation (Δ*rhcN*) was slightly affected to nodule formation except for those nodulating *Vigna radiata* cv. SUT4 and *Crotalaria juncea*. The nodule numbers and phenotype were improved by Δ*rhcN* strain. Overall, these results are likely that divergency of nod-factors in DOA9 plays as a main key for symbiosis interaction, while T3SS might trigger the plant immunity depending on plant species.

POSTER 5A-13

Improvement of the Symbiotic Performance of a Chickpea Rhizobium by Additional Copies of the *clpB* Chaperone Gene

Ana Paço[1], Clarisse Brígido[1], Ana Alexandre[1], Pedro F. Mateos[2], Solange Oliveira[1]

[1] *ICAAM-Instituto de Ciências Agrárias e Ambientais Mediterrânicas / IIFA-Instituto de Investigação e Formação Avançada, Universidade de Évora, Évora, Portugal*
[2] *Departamento de Microbiología y Genética, Centro Hispano Luso de Investigaciones Agrarias, Universidad de Salamanca, Salamanca, Spain*

The ClpB chaperone is involved in bacterial stress response. Recent studies suggest that this protein has also a role in the chickpea-rhizobia symbiosis (1). To improve the stress tolerance as well as the symbiotic performance of a chickpea microsymbiont, the *Mesorhizobium mediterraneum* UPM-Ca36T strain was genetically transformed with pPHU231 containing an extra-copy of the *clpB* gene. To investigate if the *clpB*-transformed strain displays an improved stress tolerance, bacterial growth was evaluated under heat and acid stress conditions. In addition, the symbiotic performance of the *clpB*-transformed strain was evaluated using plant growth assays (hydroponic and pot trials). The *clpB*-transformed strain is more tolerant to heat shock than the strain transformed with pPHU231, supporting the involvement of ClpB in rhizobia heat shock tolerance. Both plant growth assays confirmed that ClpB has an important role in chickpea-rhizobia symbiosis. The nodulation kinetics analysis showed a higher nodulation rate with the *clpB*-transformed strain. More remarkably, its symbiotic effectiveness increased ~60% at pH5 and 83% at pH7, compared to the wild-type strain. Furthermore, a higher frequency of root hair curling was observed in plants inoculated with the *clpB*-transformed strain, compared to the wild-type. The superior root hair curling induction, nodulation ability and symbiotic effectiveness of the *clpB*-transformed strain may be due to an increased expression of symbiosis genes. Indeed, higher transcript levels of the nodulation genes *nodA* and *nodC* were detected in the *clpB*-transformed strain. The improvement of rhizobia by addition of extra-copies of the *clpB* gene may be a promising approach to develop strains with enhanced stress tolerance and symbiotic effectiveness, thus contributing to their success as crop inoculants, particularly under environmental stresses. This is the first report on the improvement of a rhizobium with a chaperone gene (2).

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POSTER 5A-14

Investigation the Function of Some Nitrogenase Genes Located on Chromosome and Megaplasmid of *Bradyrhizobium* sp. DOA9

Jenjira Wongdee[1], Pongpan Songwattana[1], Rujirek Noisangiam[1], Nico Nouwen[2], Joel Fardoux[2], Clémence Chaintreuil[2], Nantakorn Boonkerd[1], Neung Teaumroong[1], Eric Giraud[2], Panlada Tittabutr[1]

[1] *School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand*
[2] *Laboratory of Tropical and Mediterranean Symbioses (LSTM), Campus International de Baillarguet, IRD, Montpellier, France*

Bradyrhizobium sp. strain DOA9, isolated from *Aeschynomene americana*, contains two replicons, a chromosome and a symbiotic megaplasmid. DOA9 has one copy of *nifV* which facilitate the nitrogenase activity under free-living state. Moreover, several nitrogenase genes, such as *nifH*, *nifD*, *nifK*, *nifA*, and *rpoN* were presented on both chromosome and megaplasmid. It is interesting to investigate the function of these genes whether they were redundant for nitrogenase activity in this strain. Firstly, two distinct copies of the *nifDK* genes, annotated *nifDKc* (on chromosome) and *nifDKp* (on megaplasmid) were examined the transcription using *gusA* reporter fusions. The result showed that both *nifDKc* and *nifDKp* were highly expressed under symbiosis, while *nifDKc* was predominantly respond under free-living state. Mutational analysis indicated that both *nifDKc* and *nifDKp* are required for fully function of nitrogenase activity during symbiosis with *A. americana*, while, *nifDKc* is the major contributor of nitrogenase during free-living state. However, the expression of *nifENX*, which were co-transcribed from *nifDKc* operon on chromosome is required for the function of *nifDKp*. Secondly based on mutational analyses, it was showed that *nifAc* and *rpoNc*, but not *nifAp* and *rpoNp* were required for nitrogen fixation under free-living state. However, both *rpoNc* and *rpoNp* were required for nitrogenase activity under symbiosis with *A. americana*, while *nifAc* and *nifAp* could function instead of each other under symbiosis state. These results indicate that the strain DOA9 carries the functional *nif* genes on both chromosome and megaplasmid that are contributed differently to the nitrogenase activity depending on state of bacteria (free-living or symbiosis). The regulation among these *nif* genes located on chromosome and megaplasmid will be further investigated.

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POSTER 5A-15

Is T6SS Involved in the Establishment of Symbiosis in β-rhizobia?

Samanta Bolzan de Campos, M. Lardi, L. Eberl and G. Pessi

Institute of Plant Biology, Department of Microbiology, UZH Zurich, Switzerland
samanta.bolzandecampos@uzh.ch; gabriella.pessi@uzh.ch

Burkholderia tuberum and *B. phymatum* are beta-rhizobial strains able to fix atmospheric nitrogen in the root nodules of legumes. They were recently isolated from nodules of leguminous plants in South Africa (*B. tuberum*) and French Guiana (*B. phymatum*) and are usually found associated with plants of the Mimosoideae and Papilionoideae subfamily of legumes (Fabaceae). Since the ability of *Burkholderia* strains to establish a nitrogen-fixing symbiosis with legumes has recently been discovered, the molecular mechanisms underlying this symbiotic interaction are still not understood.

The newly discovered Type VI Secretion System (T6SS) is widespread among Gram-negative bacteria and has been associated with pathogenesis playing roles in bacterial cell targeting, as well as in eukaryote cell targeting. Both *B. phymatum* and *B. tuberum* possesses at least 2 copies of the T6SS structural genes in its genome. In order to identify the function and the role of T6SS in the these endosymbiotic *Burkholderia* the gene coding for a structural component of the T6SS *tssI* was disrupted by the insertion of a plasmid, and the resulting strains were tested for the competition abilities in *in vitro* assays and *in planta* nodulation assays using *Phaseolus vulgaris* (common bean).

POSTER 5A-16

Mesorhizobium loti R7A Mutants Deficient in the Biosynthesis of GalA in Lipid A Form Normal Symbioses

Artur Muszyński[1], Richard W. Zoller[1], John Sullivan[2], Young-Tae Kim[1], Parastoo Azadi[1] and Clive Ronson[2]

[1] *Complex Carbohydrate Research Center, The University of Georgia, Athens, United States*
[2] *Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand*

The surface of a *Mesorhizobium loti* cell is a complex matrix consisting of lipopolysaccharide (LPS), capsular polysaccharides (CPS), cyclic glucans, and acidic exopolysaccharides (EPS). Due to the high complexity of the bacterial envelope, the role of these surface polysaccharides and glycolipids in the symbiotic interaction with legumes is not fully understood. However, we have recently demonstrated signaling properties of monomeric EPS in the *M. loti* – *Lotus japonicus* symbiosis (1). LPS, the outermost component of the rhizobial cell envelope, plays an important role during the symbiotic infection of root cells and differentiation of rhizobia into nitrogen-fixing bacteroids within these cells, in that it undergoes structural changes to accommodate the bacterial cell to the intracellular plant environment and to evade plant defense mechanisms (2). Structural studies of *M. loti* (MAFF303099) *and M. huakuii* Lipid A demonstrated that it consists of P→4-β-D-Glc*p*N3N-(1→6)-α-D-Glc*p*N3N-(1-1)-α-D-Gal*p*A backbone substituted with amide and ester linked fatty acids, including very long chain fatty acids (3,4). The function of the Lipid A α-(1,1)-Gal*p*A transferase (GalAT) gene, *rgtF*, was recently demonstrated by heterologous expression of MAFF303099 GalAT in *R. etli* (4); however its role in *M. loti* has not been investigated. This work aimed to better understand the function of GalAT in *M. loti*, and most importantly to determine if the acidic GalA Lipid A substituent had a role in the *M. loti* R7A symbiosis. Chemical and structural analysis of Lipid A isolated from a *rgtF* deletion mutant of strain R7A revealed complete loss of GalA substitution in comparison with Lipid A of the parent strain. The structure of LipidA was restored to that of wild-type in an *rgtF* complemented strain. Interestingly, the mutant strain still formed an effective symbiosis with the host *L. japonicus*.

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POSTER 5A-17

Molecular Basis for Negative Regulation of the *Bradyrhizobium diazoefficiens* Transcription Factor $FixK_2$

Juan J. Cabrera[1], Sergio Parejo[1], Noemí Fernández[1], Sergio Salazar[1], Mariette Bonnet[2], María J. Delgado[1], Hauke Hennecke[2], Eulogio J. Bedmar[1], Socorro Mesa[1]

[1] *Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín, CSIC, Granada, Spain*
[2] *Institute of Microbiology, ETH-Zurich, Zurich, Switzerland*

In the facultative soybean endosymbiont *Bradyrhizobium diazoefficiens*, the $FixK_2$ protein plays a key role in a complex regulatory network which controls a large number of genes required for the anoxic, microoxic, and symbiotic growth of this bacterium (1). $FixK_2$ is a CRP/FNR-type transcription factor, ubiquitous proteins which respond to a wide range of metabolic and environmental cues (2). Expression of the *fixK*$_2$ gene is activated by the superimposed two-component regulatory system FixLJ in response to microoxia (at or below 5% O_2) and repressed by its own product (directly or indirectly; 3). $FixK_2$ is also negatively regulated at posttranslational level, by the oxidation of its singular cysteine residue (Cys-183; 4) and by proteolysis, both by general degradation by the $ClpAP_1$ chaperone-protease system (5), and by specific cleavage at its C-terminal end (between Val-220 and Leu-221). Remarkably, the $FixK_2$ structure in complex with its cognate DNA revealed that the last twelve amino acids of $FixK_2$ are surface-exposed, rendering the protein accessible for specific cleavage and general degradation (6).

In order to expand our understanding of the mechanism underlying the negative regulation of $FixK_2$, on the one hand, we performed a functional mutagenesis of two transcription factor genes (bll2109, bll3466) and analyzed *fixK*$_2$ expression in microoxically-grown (0.5% O_2) *B. diazoefficiens* cells; on the other hand, we characterized a series of protein variants with modifications within the C-terminal part of $FixK_2$, specifically in the oxidation-sensitive Cys-183, and in the last twelve amino acids (truncated derivatives, proteins with amino acid exchanges at the cleavage site, and chimeric variants with similar or different predicted secondary structure). Our results showed that, while the mechanism by which $FixK_2$ exerts a negative feed-back on its own expression remains enigmatic, the C-terminal stretch of twelve amino acids plays a crucial role, not only in $FixK_2$ proteolysis but also in its DNA binding capacity and activity. Interestingly, the exchange of Cys-183 to Asp resulted in an inactive $FixK_2$ derivative which did not complement the phenotype of a Δ*fixK*$_2$ strain.

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POSTER 5A-18

New Unexpected Functions for ACC Deaminase Genes in *Sinorhizobium meliloti*

Alice Checcucci[1], E. Azzarello[4], M. Bazzicalupo[1], A. De Carlo[2], G. Emiliani[2], S. Mancuso[4], G. Pieraccini[5], G. Spini[3], C. Viti[3] and A. Mengoni[1]

[1] *Department of Biology, University of Florence, Sesto Fiorentino (Firenze), Italy*
[2] *IVALSA Centro Nazionale Ricerche, Sesto Fiorentino, Florence 50019, Italy*
[3] *Department of Agri-food Production and Environmental Science (DISPAA), GenExpress faciliUniversity of Florence, Sesto Fiorentino, Florence, Italy*
[4] *Department of Agri-food Production and Environmental Science (DISPAA), University of Florence, Sesto Fiorentino, Florence, Italy*
[5] *Centro di servizi di Spettrometria di Massa (CISM), Sesto F.no, Florence, Italy*

Plant-associated bacteria exhibit a number of different strategies and specific genes allow bacteria to communicate and metabolically interact with plant tissues. Among the genes often found in plant-associated bacteria the gene that encoding the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (*acdS*) is quite ubiquitous and it is supposed to be involved in the sequestering and cleaving of plant-produced ACC, the precursor of the plant stress hormone ethylene. In the symbiotic model species *Sinorhizobium meliloti, acdS* is present in the dispensable genome fraction. Few data are present on the role of such gene during symbiosis and plant-bacteria interaction.

To clarify this issue, an extensive phylogenetic and comparative genomic analysis of *acdS* orthologs has been performed in genomes of *S. meliloti* strains and functional studies have been carried out by expressing two phylogenetically distant *acdS* orthologs in the model strain *S. meliloti* 1021, which lacks the gene. Then, the ACC deaminase activity of recombinants vs. parental strain using standard biochemical assay have been performed. The symbiotic and endophytic phenotypes have been evaluated with respect to modulation of ethylene production by the host plant, competition for root nodule occupancy and plant colonization. Moreover, the metabolic profile determined by *acdS* for the recombinants and the wild type have been tested. Functional studies of transcriptional control by cloning of *acdS* promoter cassette in an expression vector have been performed and the activity level of the promoter expression cassette with different possible inductors evaluated.

Data showed that *acdS* orthologs present in *S. meliloti* are polyphyletic and may indeed derive from different alphaproteobacteria representatives. Different levels of ACC deaminase activity from tested *acdS* orthologs were detected, no modulation of plant ethylene levels was detected and no increase in fitness for nodule occupancy was found in the *acdS*-derivative strains compared to the parental one. Surprisingly, AcdS was shown to confer the ability to utilize formamide and some dipeptides as sole nitrogen source. The *acdS* gene activation seems not plant-specific. However, the function of *acdS* in symbiotic bacteria has not been fully clarified, especially in relation to the mutualistic behavior of rhizobial strains. We conclude that *acdS* in *S. meliloti* could be more related to the exploitation of unusual nitrogen sources, in connection with rhizospheric colonization or endophytic life-style.

POSTER 5A-19

Proteomic Analysis Reveals Host-specific Differential Expression of *Rhizobium leguminosarum* bv viciae Proteins in Pea *vs.* Lentil Bacteroids

David Durán Wendt[1], Neyser de la Torre[1], Carlos García[2], Anabel Marina[2], Tomás Ruiz-Argüeso[1], Jose Palacios[1]

[1] *Centro de Biotecnología y Genómica de Plantas (CBGP), and E.T.S.I. Agrónomos, Universidad Politécnica de Madrid. Campus de Montegancedo, Pozuelo de Alarcón, Madrid, Spain*

[2] *Servicio de Proteómica, Centro de Biología Molecular Severo Ochoa (CBMSO), CSIC-UAM, Campus de la Universidad Autónoma de Madrid, Cantoblanco, Madrid, Spain*

Some rhizobia are able to effectively nodulate several legume plants, which constitute a cross-inoculation group. In the case of *Rhizobium leguminosarum* bv viciae the cross inoculation group includes plants belonging to genera *Pisum*, *Lens*, *Lathyrus,* and *Vicia*. It has been shown that different hosts within this group modulate the expression of rhizobial traits in a host-specific manner, suggesting that different plants might impose different environments for the endosymbiont. For instance, when pea and lentil plants interact with the same *R. leguminosarum* bv viciae strain, significant differences occur in the symbiotic expression of hydrogenase (1), and also in nickel input and efflux (2,3). These data indicate that bacteria adapt differently to the conditions provided by each host. A relevant aspect of the symbiotic environment within the nodule is the presence of nodule-specific cysteine-rich (NCR) peptides produced by the plant and exported into the bacteroids (4), a phenomenon widely extended in legume plants from the IRLC (Inverted Repeat-Lacking Clade) group (5).

In this communication we present a proteomics approach to study the differential expression in bacteroids induced by *R. leguminosarum* strain UPM791 in pea and lentil plants. In each case, over 800 bacterial proteins were identified through LC-mass spectrometry analysis. Differences between bacteroids from both hosts affected a surprisingly high number of proteins. These differences are currently being quantified through iTRAQ labelling. Plant-derived NCR peptides were also identified in bacteroid extracts from both legumes. The identity of these peptides is being determined through complementary transcriptomic analysis. Preliminary data indicate that pea and lentil plants deliver to the bacteroids a mixture of common and host-specific NCR peptides.

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POSTER 5A-20

Quantity and Quality of Different Pea Cultivars depending on Rhizobia Strains

Alise Šenberga, Laila Dubova, Ina Alsiņa, Līga Zaharāne

Institute of Soil and Plant Sciences, Latvia University of Agriculture, Jelgava, Latvia

Legume growing has been recognized as a sustainable practice in agriculture. Peas (*Pisum* sp.) are one of the most efficient legumes in terms of nitrogen fixating ability. They can fix up to 190 kg ha^{-1} nitrogen per year (1). Peas are rich in protein, starch, carbohydrates, minerals and vitamins. It has been suggested that the increase of yield and protein content of peas depends on the genotype of the rhizobium strain used (2).

Two different pea cultivars ('Kelvedon Wonder' and 'Retrija') and one breeding line ('H 91 – 14 – 43') were used in this study. As the role of rhizobia is very crucial when growing legumes, research on this subject is of great importance. Therefore, in our experiments three different rhizobia strains were used – RP023, isolated from *Pisum sativum,* and two strains isolated from *Vicia faba* (RV407 and RV505). All the strains were obtained from the Rhizobia Collection of Institute of Soil and Plant Sciences. Molecular analyses showed that all of the rhizobia strains used were genetically different from each other (based on fingerprinting results, as well as on the analyses of the ITS region).

The main focus of this study was on the quality and quanity of pea yield. For pea crop quantity we looked at the yield of the peas, as this is one of the main factors that most of the legume growers are interested in. As protein is an integral part of food and fodder, protein content in peas was measured as an indicator for crop quality. The aim was to determine whether rhizobia use can change the yield and/or the total protein content of the seeds.

Results showed that the same rhizobium strain can have a significantly different effect on the yield (t ha^{-1}) between pea cultivars. While strain RV407 efficiency was almost consistent for all the pea cultivars, strain RP023 showed clear cultivar specificity. RP023 treatment showed the lowest results in 'Retrija' (even lower than control plants) and the highest yield in 'Kelvedon Wonder' and breeding line 'H 91 – 14 – 43', comparing to control plants without rhizobia treatment. Pea cultivar 'Kelvedon Wonder' showed higher yield with all of the strains compared to the control plants. Strain RV505 showed no significant impact on the yield of peas.

In this study no significant differences of total protein content were detected between cultivars as well as between rhizobia treatments. It has been suggested that legumes tend to produce as many seeds as possible, notwithstanding the available nitrogen level. This might have been done at the expense of protein content of the seed. Our study suggests that the choice of rhizobium strain for pea inoculation has to be based not only on the agroclimatic conditions, but also on the specific pea cultivar.

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POSTER 5A-21

Rhizobial Type III Effector Protein Regulates Soybean Nodulation

Sachiko Masuda, Hiroki Miwa, Michiko Yasuda, Omar M. Faruque and Shin Okazaki

Tokyo University of Agriculture and Technology, Tokyo, Japan

Leguminous plants establish symbiotic interaction with rhizobia, forming nitrogen-fixing root nodules. The perception of rhizobial Nod-factor by plant LysM receptor protein activate signaling cascade in host plant, which leads to root hair deformation and cortical cell divisions. Therefore, Nod factor is thought to be the primary determinant for root nodule symbiosis. We previously revealed that *Bradyrhizobium elkanii* USDA61 can infect plant and form nodules by the type III secretion system, independently of Nod factor signaling pathway (1). In the interaction between soybean and *Bradyrhizobium*, rhizobium colonizes on root surface and may directly infect plant independently of infection thread formation in root hair cells. However, the molecular mechanism of Nod-independent nodulation remains to be resolved. We have been analyzing type III effector proteins in this unique symbiosis.

Previously we found that cysteine protease of *B. elkanii* USDA61 is involved in soybean *Rj4* incompatibility (2). In this study, we found that *tts* box is present upstream of this cysteine protease, which is required for gene expression of Type III secretion system. In silico analysis predicted that N-terminus of this protein carried the signal peptides required for Type III secretion system. Thus this cysteine protease is thought to be Type III secretion system effector protein. Further nodulation test using soybean symbiotic mutant and the mutant of this cysteine protease revealed that this effector act as a positive regulator of soybean nodulation. Currently we are analyzing the secretion of this protein and its function in Nod-independent nodulation.

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POSTER 5A-22

The Effect of Aluminium on the Nodulation of Lucerne: A Comparison of Two Rhizobia Strains and Two Lucerne Lines

Kathryn Wigley[1], Derrick Moot[1], Hayley Ridgway[1], Alan Humphries[2], Steve Wakelin[3], Ross Ballard[2]

[1] *Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln, New Zealand*
[2] *South Australian Research and Development Institute, Adelaide, Australia*
[3] *AgResearch Ltd, Lincoln Research Centre, Lincoln, New Zealand*

A significant barrier to wider adoption of lucerne is the sensitivity of this legume to acidity, which is often correlated to high available aluminium (Al^{3+}). This affects the survival of rhizobia (1) and interferes with the legume–rhizobia symbiosis (2). Recent work in Australia has identified acid tolerant rhizobia that can increase nodulation at low pH (3). However, the effect of Al^{3+} on these strains has not been investigated. This study compared the effect of Al^{3+} on the nodulation of lucerne plants inoculated with the current commercial strain of *Sinorhizobium meliloti* used in Australia and New Zealand (RRI128) alongside an acid tolerant strain (*S. meliloti* SRDI736).

The experiment was conducted in a hydroponic system. Nodulation of lucerne by RRI128 and SRDI736 was tested at four Al levels (0, 2, 4 and 8 μM) at pH 5.1. 'Stamina 5', a cultivar sown in New Zealand, and 'TA37', a line bred in Australia with increased Al^{3+} tolerance, were compared.

The combination of Al^{3+} tolerant lucerne (TA37) inoculated with an acid tolerant rhizobia (SRDI736) produced more nodules per plant (3.3 vs. 1.0; $P<0.01$) and a higher percentage of plants nodulated (91 vs 41%; $P<0.01$) compared with TA37 plants inoculated with RRI128. Stamina 5 inoculated with SRDI736 also produced more nodules per plant (1.1 vs 0.3; $P<0.01$) and had a higher percentage of plants nodulated (46 vs. 12%; $P<0.01$) compared with Stamina 5 plants inoculated with RRI128. Overall, however, Stamina 5 had a lower percent of plants nodulated compared with TA37. As the concentration of Al^{3+} increased, nodules per plant decreased ($P<0.05$) for plants inoculated with SRDI736 and RRI128, irrespective of cultivar. However, the combination of TA37 and SRDI736 still had more ($P<0.01$) nodules per plant compared with standard lucerne/rhizobia combinations at 8 μM of Al. This confirms progress in selection of aluminium tolerant plants and rhizobial strains, and highlights potential for lucerne production in soils previously seen as having problematic levels of Al^{3+}.

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POSTER 5A-23

The Salt Shock Transcriptional Profile of *Mesorhizobium loti* MAFF303099 is Distinct from that of other Rhizobia

Marta Laranjo[1,2], Ana Alexandre[1,2], Solange Oliveira[1]

[1] *Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Évora, Portugal*
[2] *Instituto de Investigação e Formação Avançada (IIFA), Universidade de Évora, Évora, Portugal*

Mesorhizobium loti MAFF303099, which nodulates the model legume *Lotus japonicus*, was the first rhizobium to have its genome sequenced (1). To better understand the response of this large-genome bacterium to stress, we performed global transcriptional analyses following heat (2), acid (3) and salt stresses. Here we focus on the transcriptional profile of *M. loti* cells after salt shock.

Our results show that *M. loti* MAFF303099 displays a distinct salt shock transcriptional response compared to that seen in other rhizobial species. A total of 385 genes were differentially expressed following salt shock (272 overexpressed, 113 underexpressed). Previous studies on *S. meliloti* and *R. etli* reported a strong replicon bias in response to NaCl (4, 5). In contrast, our results showed that most of the genes induced or repressed in response to salt are more evenly distributed among the chromosome and two plasmids. Genes involved in biosynthesis of important osmoprotectants, such as trehalose, were not induced in *M.loti*, unlike what has been reported for other rhizobia (4, 5). Expression of *M. loti* genes encoding chaperones and proteases was not affected by salt shock, the reverse of what is seen in other rhizobia. Moreover, no sigma factors showed differential expression, while both *rpoE4* and *rpoH2* were salt-induced in *S. meliloti* and *R. etli* (4, 5).

On the other hand, many transcriptional regulators were overexpressed in *M. loti*, as also seen in *S. meliloti* (4), suggesting an important role for regulation at the transcriptional level in response to high salt. Similarly, genes encoding components of several ABC transporter systems were upregulated in both *M. loti* and *R. etli* (5), consistent with the idea that export of certain compounds may be an important way for the cell to maintain viability under salt stress. Because *M. loti* MAFF303099 showed such a distinct salt stress transcriptional response compared to that seen in other rhizobia makes this a substantial contribution to our current knowledge of the molecular bases of the bacterial salt stress response.

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POSTER 5A-24

The Symbiosis Island of NZP2037 Holds the Secrets to *Lotus* Host Specificity

James T. Chang[1,2], John T. Sullivan[1,2], Simon J. Kelly[2,3], Jens Stougaard[2,3] and Clive W. Ronson[1,2]

[1] *Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand*
[2] *Centre for Carbohydrate Recognition and Signalling, University of Aarhus, Aarhus, Denmark*
[3] *Department of Molecular Biology and Genetics, University of Aarhus, Aarhus, Denmark*

Mesorhizobium loti strains fall into two host-range groups on the basis of their ability to nodulate *Lotus pedunculatus*. Group 1 strains, including R7A, MAFF303099 and NZP2213, only induce largely uninfected nodule primordia on this host, whereas Group 2 strains, including NZP2037, SU343, NZP2014, and NZP2042, form effective nodules. All strains form effective nodules on *L. japonicus* Gifu, though with varying efficiencies. Interestingly, *exoU* mutants of Group 1 strains only induce uninfected primordia on *L. japonicus* whereas *exoU* mutants of at least most Group 2 strains strains form effective nodules on both hosts. We are investigating the genetic basis of these host-range differences and whether there is an interaction between Nod factor and exopolysaccharide perception in determining host range. Transfer of the symbiosis islands from each Group 2 strain to a non-symbiotic derivative of the Group 1 strain R7A cured of its symbiosis island yielded exconjugant strains that effectively nodulated *L. pedunculatus*, showing that the host range difference was encoded on the symbiosis island. Using comparative genomics, we have identified several *nod* genes that are present in all Group 2 strains but absent from Group 1 strains. To date, deletion of these genes individually and in some cases in pairs has not revealed the genes that determine the host-range difference. We will report further analyses of these genes and also whether the addition of *exoU* mutations affects the mutant phenotypes.

POSTER 5A-25

Using Raman Microscopy to Investigate Nitrogen Fixation Mutants of *Rhizobium leguminosarum*

Isabel U. Webb[1,2], J. Xu[4], W. Huang[4], E. James[5], E. Barclay[2], N. J. Watmough[3], P. S. Poole[1]

[1] *Department of Plant Sciences, University of Oxford, Oxford, United Kingdom*
[2] *John Innes Centre, Colney Lane, Norwich, United Kingdom*
[3] *University of East Anglia, Norwich, United Kingdom*
[4] *Department of Engineering, University of Oxford, Oxford, United Kingdom*
[5] *James Hutton Institute, Dundee, United Kingdom*

Several genes are known to be essential for bacteroid development and nitrogen fixation in *Rhizobium leguminosarum*, including an operon of four genes, fixABCX. The products of these four genes show homology to the mammalian electron transfer flavoprotein and its cognate ubiquinone oxidoreductase, suggesting a role in electron transfer. Similar roles have been shown for homologous proteins in free-living nitrogen fixing species, but their role within symbiotic nitrogen fixation is less well understood.

This work aims to understand control of fixABCX gene expression and characterize the role of the FixABCX proteins. Bacteroids isolated from plants inoculated with the deletion strains show striking differences in morphology to those from wild type strains at both whole nodule and cellular level. This has been observed using transmission electron microscopy and demonstrates an effect on other metabolic pathways within the bacteroids. Two distinct strategies can be identified to deal with the redox changes imposed when nitrogen fixation is knocked out; production of polyhydroxybutyrate, and production of poly-phosphate.

Raman microscopy (1-2) has been used to confirm the identity of metabolites in these *fix* mutants, along with other mutants in *R. leguminosarum*. Raman microscopy allows investigation of morphological changes at a single-cell level, and has allowed new insights into the functions of key genes in *R. leguminosarum*.

This work was supported by a BBSRC DTP studentship based at the University of Oxford, the John Innes Centre and the University of East Anglia.

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POSTER 5A-26

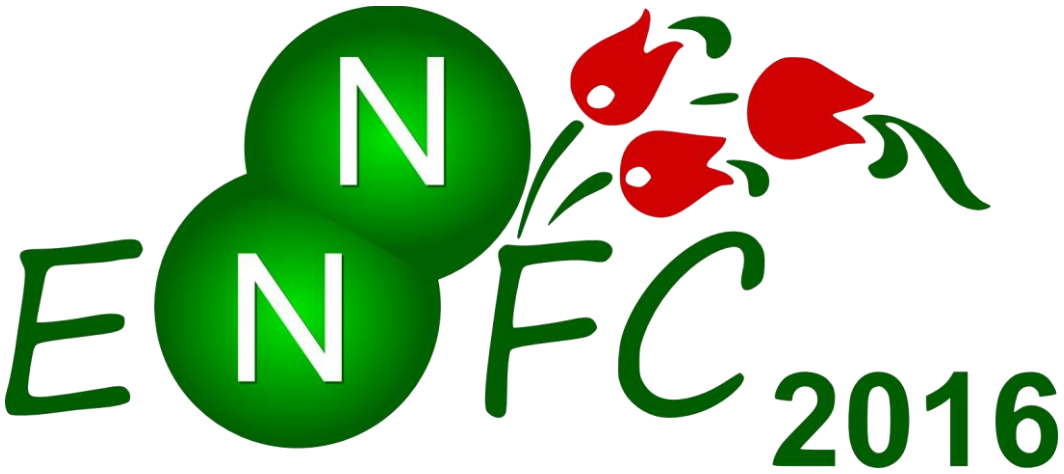
β-rhizobial Symbiosis: New Insights from Genome-wide Transcriptome and Proteome Analysis

Martina Lardi, Y. Liu and G. Pessi

Institute of Plant Biology, Department of Microbiology, UZH Zurich, Switzerland
martina.lardi@uzh.ch; gabriella.pessi@uzh.ch

Various β-proteobacteria of the genera *Cupriavidus* and *Burkholderia* have been recently been shown to be able to establish nitrogen-fixing symbiosis with legumes. At present, very little is known about the molecular determinants underlying the successful symbiosis between legumes and the so called β-rhizobia.

In this study, we use RNA-Sequencing and Shotgun-Proteomics to investigate and compare the expression profiles of *Burkholderia phymatum* grown in free-living conditions and inside *Phaseolus vulgaris* root nodules. This genome-wide expression study on β-rhizobial root nodules confirmed that genes and proteins coding for known important symbiotic functions such as nitrogen fixation and hydrogenase were significantly up-regulated during symbiosis. In addition, genes coding for flagella and type VI secretion system showed decreased expression in nodules suggesting that these functions are not needed for life inside the plant. Currently, we are investigating several genes and proteins highly expressed and up-regulated in *P. vulgaris* root nodules for their potential role in symbiosis. We are particularly interested in the identification of transcriptional regulators important for an efficient symbiosis. Preliminary studies showed that beside NifA, another σ^{54} activator is playing an important role inside *P. vulgaris* root nodules.



POSTER SESSION 5B

Functioning of the N-fixing Symbioses /Plants/

Room Mercure

POSTER 5B-1 /LIGHTNING TALK/

The Role of U-box Ubiquitin Ligases during Plant-Microbe Interactions

Ernö Kiss, Boglárka Oláh, Zoltán Bozsóki, Andrea Borbola, Erzsébet Fehér-Juhász, Sándor Jenei and Gabriella Endre

Biological Research Centre of the Hungarian Academy of Sciences, Institute of Genetics, Szeged, Hungary

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Post-translational modifications represent very rapid and efficient systems in the precise regulation of the function of different proteins. Ubiquitination of proteins is a modification that can significantly change the fate of a protein. It can target the protein for degradation to the proteasome or can change its activity or location within the cell either. The E3 ubiquitin ligases - one of the three enzymes needed for linking the ubiquitin to the protein targets - are of particular interest in this process as they confer substrate specificity, but also, they can identify several target proteins for ubiquitination. In plants, the U-box domain containing E3 family has undergone a large gene expansion that may be attributable to biological processes unique to the plant life cycle. Research on these genes from several different plants has started to elucidate a range of functions for this family, from self-incompatibility and hormone responses to defence and abiotic stress responses, and they also could be identified as being essential for nitrogen-fixing symbiosis.

We work on the characterization of two different kinds of U-box containing E3 ligases that have roles in the process of nitrogen-fixing symbiosis in *Medicago truncatula*. Beside the functional U-box driving the link from the E2 enzyme onto the target protein, they contain ARM-repeats, but the rest of the modules are different. Their E3 ligase activity has been confirmed in biochemical experiments, on the other hand their in vivo detection and examination is very difficult due to their unstable nature. In symbiotic plant tests we have characterized their mutant phenotype as well as the effect of their over-expressions. Their homologs from other plants were also recognized and cloned. When truncated form of these E3 ligases or their homologs were introduced into the mutant or wild type plants, the resulting phenotype allowed to distinguish the possible function of the different domains.

POSTER 5B-2 /LIGHTNING TALK/

MtNramp1, MtZIP6, and MtCOPT1 are Respectively Responsible for Iron, Zinc, and Copper Uptake by *Medicago truncatula* Nodule Cells

Manuel Tejada-Jiménez[1], Ángela Saéz[1], Marta Senovilla[1], Rosario Castro-Rodríguez[1], Isidro Abreu[1], Viviana P. Escudero[1], Igor Kryvoruchko[2], Mercedes M. Lucas[3], Michael Udvardi[2], Juan Imperial[4], Manuel González-Guerrero[1]

[1] *Centro de Biotecnología y Genómica de Plantas, Campus de Montegancedo, Universidad Politécnica de Madrid, Madrid, Spain*
[2] *Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, United States*
[3] *Instituto de Ciencias Agrarias, Consejo Superior de Investigaciones Científicas, Madrid, Spain*
[4] *Consejo Superior de Investigaciones Científicas, Madrid, Spain*

Legume nodules require relatively large amounts of transition metals (iron, copper, zinc…) as cofactors of some of the key enzymes involved in symbiotic nitrogen fixation (1). These metals are provided by the host plant through the vasculature and, in the case of indeterminate type nodules, released in the infection/differentiation zone of the nodule (2). Consequently, a number of metal transporters must be in place to introduce these elements into the nodule cells to be later delivered to the symbiosomes.

We have identified three *Medicago truncatula* metal transporters encoding genes, *MtNramp1* (3), *MtZIP6*, and *MtCOPT1*, that are expressed at high levels in the nodule zone where metals have to be incorporated from the apoplast. Yeast complementation assays show that they are respectively involved in iron, zinc, and copper transport towards the cytosol. Consistent with a role in metal uptake from the apoplast, they are located in the plasma membrane of nodule cells. *Knock out* or *knock-down* plants of each gene have reduced growth and reduced nitrogenase activity. These phenotypes were restored by reintroducing a wild type form of the mutated gene or by watering the plants with a nutritive solution fortified in the corresponding transported metal. These results are consistent with a model in which MtNramp1 is responsible for Fe^{2+} loading of nodule cells, while MtZIP6 is for Zn^{2+} uptake, and MtCOPT1 for Cu^{+}.

This work was supported by ERC Starting Grant (ERC-2013-StG-335284) and MINECO Grant (AGL-2012-32974) to MGG.

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POSTER 5B-3 /LIGHTNING TALK/

Thioredoxin 1 s1 is Essential for Bacterial Terminal Differentiation in the Nitrogen-fixing Symbiosis in *Medicago truncatula*

C. Ribeiro Werner[1], F. Baldacci-Cresp[1], O. Pierre[1], M. Larousse[1], G. Alloing[1], P. Mergaert[2], P. Gamas[3], N. Rouhier[4], F. Montrichard[5], P. Frendo[1] and Eric Boncompagni[1]

[1] *Institut Sophia Agrobiotech, Université de Nice-Sophia Antipolis, Sophia Antipolis, France frendo@unice.fr*
[2] *Institute for Integrative Biology of the Cell, CNRS, Gif-sur-Yvette, France*
[3] *Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR441, Castanet-Tolosan, France*
[4] *Interactions Arbres-Microorganismes, UMR1136, Université de Lorraine, Vandoeuvre-lès-Nancy, France*
[5] *Institut de Recherche en Horticulture et Semences, Université d'Angers, Angers, France*

Leguminous plants are associated with soil nitrogen-fixing bacteria to develop root nodules in which atmospheric nitrogen (N_2) is reduced to ammonium used by the host plant. In *Medicago truncatula*, the bacteria undergo a terminal differentiation into N_2-fixing bacteroids under the control of defensin-like nodule-specific cysteine-rich peptides (NCRs) produced by the host plant (1). The redox state of NCRs is important for their biological activity. Amongst the proteins involved in the regulation of redox state, thioredoxins (Trx) play key roles in the redox regulation of target proteins through the reduction of protein disulphide bonds. A nodule-specific Trx type called Trx s has been detected in *M. truncatula* (2). We showed that *M. truncatula* genome contained four *Trx s* amongst which *MtTrx s1* and *s3* were induced in the nodule infection zone. Analysis of the Trx s1 cellular localization in nodules using Trx s1::GFP translational fusion and immunolocalisation showed that Trx s1 is targeted to the symbiosome, the nitrogen-fixing organelle. Silencing of *Trx s1* using RNAi constructs reduces the nitrogen-fixation efficiency, reduced bacteroid size and impaired bacteroid DNA endoreduplication. The observation that Trx s1 is mainly expressed in nodule infection zone and localized in symbiosomes led us to postulate that NCRs may be Trx s1 substrates. Both NCR247 and NCR335 were found to interact with Trx s1 and we showed that Trx s1 expression enhanced the cytotoxic effect of NCR335 in *S. meliloti*. In conclusion, our results show that the plant partner modifies the redox state of NCR peptides and induces the bacterial terminal differentiation using specific thioredoxins.

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POSTER 5B-4 /LIGHTNING TALK/

Genetic Dissection of Nodulation Signalling using the *LORE1* Insertion Mutant Collection

Anna Malolepszy, Terry Mun, Yasuyuki Kawaharada, Niels Sandal, Dorian F. Urbanski, Jens Stougaard and Stig Uggerhøj Andersen

Centre for Carbohydrate Recognition and Signalling, Aarhus University, Aarhus C, Denmark

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With more than 640,000 annotated insertions, the non-transgenic *LORE1* insertion mutant resource offers knock-out alleles of the majority of active genes in the legume *Lotus japonicus* (http://lotus.au.dk). A detailed characterization of the *LORE1* population, including insertion preferences, transcriptional effects of insertion and estimates of background mutation rates will be presented. In addition, examples of use of the *LORE1* resource in characterization of nodulation signalling will be presented.

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POSTER 5B-5 /LIGHTNING TALK/

The Profile of NCR Peptides Produced by the Legume Host Correlates with the Morphotype of the Bacteroids

Jesús Montiel Gonzalez, Attila Szűcs, Éva Kondorosi and Attila Kereszt

Biological Research Center of the Hungarian Academy of Sciences. Szeged, Hungary

Within the nitrogen fixing root nodules, bacteroids can be reversibly or terminally differentiated depending on the legume host. Terminal differentiation is characterized by cell enlargement, genome amplification and loss of reproductive ability. *Medicago truncatula* and closely related species from the inverted repeat-lacking clade (IRLC) control terminal differentiation of bacteroids with the production of nodule-specific cysteine-rich (NCR) peptides (1). Recently, we characterized terminal differentiation of bacteroids in other six legumes that represent distinct genus from the IRLC (2). Interestingly, the degree of cell elongation was rather variable and resulted in different morphotypes: swollen (S), elongated (E), spherical (SP) and elongated-branched (EB). These findings let us to further explore bacteroid differentiation and NCR acquisition in ten IRLC legumes. For this purpose, nodule transcriptomes from six IRLC legumes were sequenced, and analyzed together with genome/transcriptome data available from another four IRLC species. *NCR* genes were identified in all of these species, however the number of *NCR* genes was considerably different, ranging from 6 NCRs in *Glycyrrhiza uralensis* up to several hundreds in *Medicago* species. A positive correlation was found between the number of the expressed *NCR*s and cell growth of the bacteroids. Legumes governing EB-bacteroid development (*Galega orientalis*, *Pisum sativum* and *Medicago* species) evolved the largest NCR families. In contrast, legumes with S-bacteroids have the lowest number of *NCR* genes. In addition, both the percentage and expression of cationic NCR were higher in legumes with EB-bacteroids compared to IRLC legumes accommodating S-, SP- or E-bacteroids. The analysis of the transcriptome and genome data from 10 species of the IRLC strongly suggests that NCR are directly implicated in cell-elongation of the bacteroids and particularly, the cationic NCR seems to be involved in the EB-morphotype. This notion is supported by the expression pattern of anionic and cationic NCR in the different zones of *M. truncatula* nodules. In zone I, 10% of the NCR transcripts are cationic, however in the interzone and nitrogen-fixing zone they reach 45% and 39%, respectively. These data provide further insights into the evolutionary process that led to NCR acquisition and terminal differentiation of the bacteroids. Several attempts are in progress to understand the emergence and diversification of NCR, as well as their role in shaping bacteroids morphotype.

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POSTER 5B-6 /LIGHTNING TALK/

Identification of Novel Symbiotic Plant Genes with the Help of *M. truncatula Tnt1* Insertional Mutants

Szilárd Kovács, Ernö Kiss, Boglárka Oláh, Sándor Jenei, Erzsébet Fehér-Juhász and Gabriella Endre

Biological Research Centre, HAS, Institute of Genetics, Szeged, Hungary

A very efficient way of identifying important genes in model plants is the forward and reverse genetic analyses of mutants. The use of tagged mutant collections has already proved to be successful in revealing plant genes that function in the nitrogen-fixing symbiosis. In this work we have used the *Tnt1* insertion mutant collection of the model legume *M. truncatula* cv. Jemalong that was produced during the EU GLIP project (www.eugrainlegumes.org) in parallel to the one already existing at the Noble Foundation for ecotype R108 (http://bioinfo4.noble.org/mutant/). In the case of the GLIP collection, during the EU project only the construction of the mutant lines was financed. Consequently, only a limited number of mutants were characterized in this collection, despite the value of such characterized collections for the community. Now we have done a large scale symbiotic screen using this mutant collection and those lines were selected, in which individuals with impaired symbiotic phenotype appeared. We focused on those mutants that indicated defects in different steps of the symbiotic process, thus 24 lines were chosen for back-cross and further genetic analyses. From these lines segregating populations were produced for 11 mutants. In the meantime, FST sequences belonging to these lines were also carefully analyzed to use candidate gene approach. Thorough phenotype characterization and genotype determination of the candidate genes resulted in the identification of the mutated gene responsible for the symbiotic phenotype in two different mutant lines. The characterization of these two genes and their protein products is in progress and will be presented.

POSTER 5B-7 /LIGHTNING TALK/

What Defines and Regulates Nodule Identity and Organogenesis?

Katharina Schiessl, Jodi Lilley, Giles Oldroyd

John Innes Centre, Colney Lane, Norwich, United Kingdom

Nodules are lateral organs that develop at the differentiation zone of legume roots in response to successful infection with symbiotic nitrogen fixing rhizobial bacteria. While studies on the symbiosis signalling pathway that leads to successful infection have identified many components of this pathway, little is known about the downstream gene regulatory networks that define nodule organ identity and direct nodule organogenesis.

Nodules emerged about 60 Mio years ago and are considered as a recent development in evolutionary terms (Sprent, 2001). Therefore, it has been hypothesised that nodules derived from lateral roots and have been modified in morphology and function to host the nitrogen fixing bacteria. Accordingly, it has recently been shown that several known key regulators of lateral root development such as members of the *PLETHORA* family play an important but modified role during nodule organogenesis (Franssen et al., 2015). However, there must also be novel genes that provide nodule identity and direct the unique development of these structures.

Here, we study nodule organogenesis in the model species *Medicago truncatula* which forms indeterminate nodules with a characteristic zonation consisting of an apical meristematic region, a transition zone where cells undergo endoreduplication, an infection zone and a nitrogen fixation zone in the central regions. In comparison to lateral root primordia that develop a central vasculature, nodules develop two or more vascular strands running along the periphery of the egg shaped nodule body, suggesting that vascular development plays a key role in nodule identity.

We aim to investigate the gene regulatory network that directs nodule identity and nodule organogenesis. In a first step, we used expression analysis with high spatial and temporal resolution on spot inoculated root sections and induced lateral roots. Comparative RNA-Seq experiments identified a set of differentially expressed genes between nodule and lateral root organogenesis over time course of development. This is be followed by detailed expression studies and functional analysis of key candidate genes. We hope that from these analyses we may be able to define the genes that provide the unique nodule identity, as well as better defining how lateral root developmental programmes underpin this process.

POSTER 5B-8

A System's Approach to Analyse Salt Stress Tolerance in *Casuarina glauca* and the Contribution of Symbiotic *Frankia* Bacteria

Ana I. Ribeiro-Barros[1, 2], Inês Graça[1], Tiago Jorge[2], Paula Alves[1], Katharina Pawlowski[3], Carla António[2], José C. Ramalho[1]

[1] *PlantStress&Biodiversity, Linking Landscape, Environment, Agriculture and Food (LEAF), Instituto Superior de Agronomia (ISA), Universidade de Lisboa (ULisboa), Lisboa, Portugal*

[2] *Plant Metabolomics Laboratory, ITQB NOVA, Instituto de Tecnologia Química e Biológica António Xavier, Portugal*

[3] *Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, Sweden*

Soil salinization is a major land degradation problem and is increasing steadily in many parts of the world. It is estimated that the continuous salinization of arable land, associated with low rainfall, high evaporation, saline irrigation water and poor water management, will result in losses of 30% of agricultural land over the next 25 years, increasing up to 50% by 2050 (1). Thus, salinity is one of the most important challenges for agricultural systems.

The actinorhizal tree *Casuarina glauca* tolerates extreme environmental conditions, such as high salinity. This species is also able to establish a root-nodule symbiosis with N_2-fixing bacteria of the genus *Frankia*. In order to analyse the mechanisms underlying salt stress tolerance *C. glauca*, we have examined the impact of increasing NaCl concentrations (200, 400 and 600 mM) in leaves and nodules of symbiotic and non-symbiotic plants. The symbiosis with *Frankia* Thr was turned off at 200 mM NaCl (2). Even so, the first stress symptoms (e.g. leaf chlorosis and necrosis) were observed only at 600 mM NaCl in both plant groups (3). The innate salinity tolerance was connected with photosynthetic adjustments, membrane preservation as well to a controlled oxidative state environment (2,4).

Analysis of the branchlets transcriptome by NGS identified ca. 180,000 contigs, with a great diversity of functions, including stress-related-, metabolic-, transport- and regulatory genes, with a marked differential expression at 400 and 600 mM NaCl. LCMS/MS and SWATH analysis identified 357 proteins, of which ca. 100 were differentially expressed in the presence of salt. Stress-responsive proteins were mainly associated with energy, amino acid and carbohydrate metabolism. GC-TOF-MS metabolite profiling of branchlet tissues revealed 26 primary metabolites, ranging from sugars to amino and organic acids, most of which decreased under stress conditions. Altogether, high-throughput analyses are in line with the morphological, physiological and biochemical analysis, reflecting the strong capacity of *C. glauca* to cope with salt stress.

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POSTER 5B-9

Agrobacterial Tumors as Possible Triggers of AON (Autoregulation of Nodulation) Suppressing Nodule Development

Alena P. Samorodova, Varvara E. Tvorogova, Maria A. Lebedeva, Ludmila A. Lutova

Department of Genetics and Biotechnology, Saint-Petersburg State University, Saint-Petersburg, Russia

Components of CLAVATA (CLV) system, such a CLV1-like kinase, CLV2 and nodulation-specific CLE peptides are known to be the parts of AON (autoregulation of nodulation) that is triggered by developing nodules and negatively regulates subsequent nodule development by a feedback mechanism. Besides that, component of CLV systems have a key role in apical meristems, where they regulate *WOX* family expression in organizing centers. Recently, we found that *WOX* genes, in particular, the *WOX5* gene, are also expressed in nodules and tumors induced by *Agrobacterium tumefaciens* on pea hypocotyls. Moreover, the expression of *WOX5* in developing nodule was increased in mutants defected in AON, indicating that AON may target *WOX5* expression in nodules.

Based on these findings, we hypothesized that both nodules and agrobacterial tumors may be regulated by and may trigger the same components of AON, including the same *WOX* and *CLV* genes. First, to estimate if agrobacterial tumors may be regulated by AON we compared tumor development in wild type pea plants and in *sym29* and *sym28* mutants defected in AON. No significant difference between the diameter of agrobacterial tumors induced by *A. tumefaciens* C58 strain in wild type pea plants and *sym29* and *sym28* mutants was found. This result indicates that AON components CLV1-like kinase (PsSYM29) and CLV2 (PsSYM28) are unlikely to regulate the size of agrobacterial tumors. Next, we suggested that developing agrobacterial tumors themselves may trigger the component of AON and thus may affect the number of nodules. Indeed, according to our results, pea plants with agrobacterial tumors that were induced prior to rhizobial inoculation have reduced number of nodules, and this effect is absent in pea mutant *sym29* defected in CLV1-like kinase, a key component of AON. This suggests that agrobacterial tumors may produce a signal activating CLV1-like kinase and thereby suppress nodule number. To check if CLE-peptides may represent such a signal we are planning to estimate expression levels of a set of CLE genes in agrobacterial tumors, including nodulation-specific CLE genes.

Together, our data suggest that agrobacterial tumors avoid the regulation by AON, however, agrobacterial tumors may trigger the AON suppressing subsequent nodule development.

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POSTER 5B-10

Analysis of a Cysteine-rich Receptor-like Protein Kinase Required for the Effective Symbiotic Interaction between *Medicago truncatula* and *Sinorhizobium meliloti*

Anikó Gombár[1], Á. Domonkos[1], B. Horváth[1], G. Kováts[1], K. Miró[1], G. Iski[1], F. Ayaydin[2], K. Bóka[3], P. Kaló[1]

[1] *Agricultural Biotechnology Institute, NARIC, Gödöllő, Hungary*
[2] *Cellular Imaging Laboratory, Biological Research Center, Szeged, Hungary*
[3] *Eötvös Lorand University, Department of Plant Anatomy, Budapest, Hungary*

The symbiotic association between *Medicago truncatula* and *Sinorhizobium meliloti* results in the formation of nitrogen-fixing nodules. In the symbiotic nodules, rhizobia differentiate into bacteroids which reduce atmospheric nitrogen. In order to identify plant genes required for rhizobial invasion, bacteroid differentiation and effective functioning of the symbiotic nodule, *Medicago truncatula* mutants with ineffective (Fix-) symbiotic phenotype were identified and characterized. The genetic analysis revealed a new allele of the formerly identified *dnf5* mutant. The ineffective *dnf5-2* mutant developed small white nodules without the characteristic zonation of indeterminate nodules. Light and electron microscopic analysis of the invasion of the symbiotic cells host non-elongated rod shaped bacteria and the failure of bacteroid differentiation in the *dnf5-2* mutant. To identify the gene impaired in the *dnf5-2* mutant, positional cloning experiments has been initiated that identified a gene encoding a receptor-like protein kinase with a cysteine rich domain (*NCK* –nodule specific cysteine rich receptor like kinase). Complementation of the *dnf5-2* mutant with the wild-type *NCK* gene restored the symbiotic phenotype. In order to identify the sub-cellular localization of NCK, we prepared constructs of the *NCK* gene driven by its native promoter or promoters of symbiosis specific genes with high expression level. To analyse the requirement of the cysteine residues of NCK, we substituted the one or more cysteines to serines and tested the complementation capacity of the modified NCK proteins.

POSTER 5B-11

Co-inoculation with Rhizobia and Plant Growth Promoting Bacteria Improve Nodule Occupancy and Grain Production in Lentil

Macarena Gerding[1], Matías Gajardo[1], Pía Oyarzúa[1], Kianyon Tay[2], Geraldine Astroza[1] and Lambert Bräu[3]

[1] *Facultad de Agronomía, Universidad de Concepción, Chillán, Chile*
[2] *Instituto de investigaciones Agropecuarias INIA, Centro Regional de Investigación Quilamapu, Chillán, Chile*
[3] *School of Life and Environmental Sciences, Deakin University, Burwood, Australia*

Lentils (*Lens culinaris*) have been traditionally produced in the Mediterranean region of central Chile, particularly in areas subject to soil erosion and infertility. Under these conditions, nodulation is irregular and grain yields are well below standard. Rhizosphere bacteria can improve nodulation through different mechanisms, such as lowering the ethylene levels through ACC-deaminase production or by producing phytohormones (1, 2). The aim of this work was to assess the symbiotic performance of native rhizobial strains in co-inoculation with plant growth promoting bacteria (PGPB) in the field. The trial was set up at the INIA experimental station Santa Rosa. Seeds of the cultivar Araucana INIA were sown after the first autumn rains and were inoculated with the effective *Rhizobium leguminosarum* strains: Lc-2, Lc-7 and Lc-10, isolated from different soils in the Chilean Mediterranean drylands. Some of the plots were co-inoculated with *Pseudomonas* sp. strain AG-54 able to produce indole acetic acid and with the *Bacillus* strain AG-70, an ACC-deaminase producer. Nodulation and rhizobia nodule occupancy were assessed monthly during the first 90 days. Nodule occupancy was examined through re-isolation and PCR fingerprinting. Plants were harvested after 33 weeks and plant biomass, nitrogen content and grain yield were assessed. Nodule numbers and rhizobial occupancy were significantly improved when rhizobia Lc-7 and Lc-10 were co-inoculated with *Pseudomonas* AG-54 and *Bacillus* AG-70. Resident rhizobia were predominant in nodules (>65%) when plants were inoculated with rhizobia only, suggesting that PGPB play a positive role in rhizobial root infection. Grain yield and fodder nitrogen content were significantly improved when plants were inoculated with Lc-10 and both PGPB strains ($P \leq 0.05$), improving grain yields in 25% in comparison to uninoculated treatment and to inoculation with rhizobia only. In conclusion, co-inoculation of rhizobia and PGPB can improve nodulation and nitrogen fixation in lentil. This results also highlight the importance of initial nodulation and rhizobial nodule occupancy, to overcome competition and thus improve overall symbiotic performance. This research was carried out thanks to the financial support of the Fondo Nacional de Desarrollo Científico and Tecnológico, Project n° 11130479.

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POSTER 5B-12

Effect of Heavy Metal Stress on the *Medicago* - *Ensifer* Symbiosis: Analysis of Cultivars and Strains with Different Sensitivities to Cadmium

Victoria Lara-Dampier[1], Beatriz Pallol[1], Teodoro Coba de la Peña[1], Laura Barrios[2], José J. Pueyo[1], M. Mercedes Lucas[1]

[1] *Instituto de Ciencias Agrarias, CSIC, Madrid, Spain*
[2] *Secretaría General Adjunta de Informática, CSIC, Madrid, Spain*

Pollution due to heavy metals is an ever-increasing problem worldwide. Cadmium (Cd) is one of most hazardous contaminants of soils and negatively affects crop yields, and generates agri-food risks. Cd enters the environment mainly through the metallurgical industries, waste incinerators and land applications of sewage sludge and of chemical fertilizers. Legumes like *Medicago sativa* and *M. truncatula* have been proposed for phytoremediation of soils contaminated with heavy metals. Nodulated legumes with rhizobial bacteria represent an interesting potential tool in soil bioremediation; besides symbiotic nitrogen fixation, they present the advantage of integrating microorganisms that may influence metal bioavailability. Therefore, the identification of tolerant symbiotic partners and the understanding of response mechanisms of the symbiosis have notable interest.

In this work we investigated the effect of Cd stress on nodulation of a tolerant *M. truncatula* cultivar and a sensitive one inoculated with two rhizobial strains with different sensitivity to Cd, the reference strain 2011 and one strain isolated from a mining soil (1). Germinated seeds were inoculated at sowing and grown in the presence of 0, 5 or 10 μM Cl_2Cd for 22 days. The effect of the factors plant, bacteria and Cd, and their interactions on plant growth and nodulation were analysed. The high Cd treatment had a negative effect on plant growth and nodulation (delay of nodulation, decrease of the number of the nodulated plants and nodules/plant). In plants subjected to the intermediate Cd treatment, significant differences were found among the partner combinations. Our results suggest that the tolerant symbiotic partners could act synergistically to alleviate the effects of cadmium on nodulation. This information will be useful to understand Cd-tolerance and to evaluate suitability of this legume-*Rhizobium* symbiosis for phytoremediation purposes.

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POSTER 5B-13

Identification and Characterization of a Gene Family Encoding NCR Peptides in Pea (*Pisum sativum* L.)

Marina S. Kliukova[1,2], Vladimir A. Zhukov[1], Alexander I. Zhernakov[1], Olga A. Kulaeva[1], Igor A. Tikhonovich[1,2]

[1] *All-Russia Research Institute for Agricultural Microbiology, St.-Petersburg, Russia*
[2] *Saint-Petersburg State University, St.-Petersburg, Russia*

Legumes are known to establish symbiosis with nitrogen-fixing soil bacteria (rhizobia). As a result nodules are formed on the roots of the host plant. In this symbiotic organ bacteria undergo differentiation into symbiotic form - bacteroides. In leguminous plants belonging to IRLC (Inverted Repeat-Lacking Clade), such as *M. truncatula, P. sativum, V. faba* etc., bacteria are terminally differentiated and are not viable after extraction from the nodule. In a model plant *Medicago truncatula* NCR (Nodule-specific Cysteine Rich) peptides are factors causing the irreversible process of differentiation of rhizobia within the nodule (1, 2). Genome of *M. truncatula* contains about 600 genes arranged into a number of clusters. However, only half of them are actively expressed in the nodules (3). NCR peptides are highly variable short (30-60 amino acids in length) secreted polypeptides. Previously, eight nodule-specific proteins of pea (*Pisum sativum* L.) were identified as NCR-peptides (4). The aim of this study is to describe and characterize the gene family of NCR peptides in pea.

Candidate genes search was performed in nodule transcriptome assembled from 12-days old nodules of pea line SGE using tBLAST and tBLASTn algorithms (5). The search was carried out using known sequences of NCR genes of *M. truncatula* and *P. sativum* as queries. Length (35 nucleotide) and identity percent (60%) of similar fragments were chosen as criteria for selection of sequences for the further analysis. Only amino acid sequences containing a conservative cysteine motif were classified as NCR peptides. To date, we have identified more than 200 sequences that satisfy all the requirements inherent for the NCR gene family. All NCR peptides fall into two groups depending on number of conserved cysteine residues (group A with four and group B with six cysteines). We assume that additional candidate gene sequences can be found in transcriptomes of nodules collected at later developmental stages.

The future plans include search for candidate genes among all available transcriptome databases for more complete description of the gene family and to assess polymorphism of NCR gene sequences among the different genotypes of pea. It is also planned to analyze the differential expression of NCR genes in pea mutants at different stages of symbiosis using RNAseq and real-time PCR.

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POSTER 5B-14

Keeping Nodules in Check: Interplay of Rhizobial and Host Factors Controlling Nodule Morphogenesis and Integrity in Soybean?

Jean-Malo Couzigou, Raphael Ledermann, Julia Vorholt and Hans-Martin Fischer

ETH Zürich, Institute of Microbiology, Zürich, Switzerland

Legume plants interact symbiotically with soil bacteria, collectively known as rhizobia, resulting in the formation of specialized symbiotic organs, the root nodules. While rather deep molecular insights into plant-rhizobia recognition, early nodule organogenesis, and regulation of nodulation and nitrogen fixation are available to date, much less is known about the mechanisms for maintenance of nodule integrity and the origin of the underlying morphogenetic program. Only few flowering plants have evolved the unique capacity to form specialized symbiotic structures, probably through a relatively late event in the evolution of land plants, likely by co-opting pre-existing developmental pathways. Yet, it is currently unknown how the "predisposition" for nodule organogenesis was gained at the molecular level. Recently, *NOOT BOP COCH LIKE* (*NBCL*) genes of *Medicago truncatula* and *Pisum sativum* were found to ensure indeterminate nodule integrity by repressing ectopic root formation (1). This indicated that nodule vascular elements and roots might be considered as homologous structures (2, 3). Interestingly, soybean (*Glycine max*) nodules elicited by a *Bradyrhizobium diazoefficiens* mutant lacking the general stress response regulators EcfG or PhyR also showed ectopic roots, which points to a bacteria-plant signalling system that is crucial for nodule persistence and integrity (4). Recently, we started out to identify the molecular basis in both symbiotic partners that participate in the maintenance of determinate nodule identity using the *B. diazoefficiens* – soybean model. The roles of *G. max NBCL* genes during nodulation are currently being under investigation. We aim to combine cell biology, transcriptomics and metabolomics to unravel which cells, pathways and metabolites participate in the "nodule integrity checkpoint system" by comparing wild type- and *ecfG* mutant-elicited nodules. Altogether, this may provide new insights into the crucial question how a specific group of plants has acquired the ability to form nitrogen-fixing organs, which is relevant also in the light of ongoing attempts to engineer non-legume plants into rhizobial hosts.

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POSTER 5B-15

Molecular-genetic and Physiological Analysis of Senescence of Pea (*Pisum sativum* L.) Symbiotic Nodules

Tatiana Serova, Viktor Tsyganov

All-Russia Research Institute for Agricultural Microbiology, St.-Petersburg, Russia

The studies of the aging of nitrogen-fixing nodules are of the great interest due to possible important practical application. The delay of nodule senescence could improve the crop yield by limiting the use of chemical fertilizers.

In this study the wild-type SGE and a series of symbiotic mutants SGEFix$^-$-1 (*sym40*), SGEFix$^-$-3 (*sym26*) and SGEFix$^-$-7 (*sym27*) characterized by the premature degradation of symbiotic structures and early senescence of nodules were used (1, 2).

The genes encoding cysteine (*PsCyp1*, *PsCyp15a*) (3, 4) and thiol (*PsTPP*) (5) proteases, transcription factor bZIP (*PsATB2*) (6), gibberellin 2-β-hydroxylase (*PsGAOx2*) (4, 7), ACC synthase and oxidase (*PsACS2*, *PsACO1*) (8, 9) and aldehyde oxidase (*PsAO3*) (10) were selected as the marker 'senescence genes' of pea nodules.

An evaluation of transcript abundance of all selected genes was shown via real-time PCR during the aging of nodules of wild-type and mutant lines. In 4 week after inoculation mRNA levels of all analyzed genes were significantly higher in early senescence mutant nodules than in the active nitrogen-fixing wild-type nodules.

To identify expression pattern of tested 'senescence genes' in infected cells from nitrogen-fixation and senescence zones of wild-type and SGEFix$^-$-7 (*sym27*) nodules laser capture micro-dissection was carried out. The enhancement of *PsCyp15a, PsTPP, PsATB2, PsGAOx2, PsAO3* and *PsACO1* expression levels were observed with an increase in degradation degree of nodule cells.

Immunolocalization of gibberellic acid using laser confocal microscopy was performed for all analyzed genotypes. The intensity of GA labeling was decreased in the senescence zone of wild-type and mutant nodules.

Thus, the positive regulation of pea nodule senescence with ethylene and abscisic acid, the active effect of cysteine and thiol proteases, and transcription factor bZIP in nodule aging were demonstrated. The possible negative regulation of pea nodule senescence with gibberellic acid was also shown.

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POSTER 5B-16

MtMOT1.3 Mediates Molybdenum Transport to Rhizobia-infected *Medicago truncatula* Nodule Cells

Manuel Tejada-Jiménez[1], J. Imperial[2], M. González-Guerrero[1]

[1] *Centro de Biotecnología y Genómica de Plantas. Universidad Politécnica de Madrid. Pozuelo de Alarcón, Madrid, Spain*
[2] *Consejo Superior de Investigaciones Científicas. Madrid. Spain*
manuel.gonzalez@upm.es

Symbiotic nitrogen fixation (SNF) requires molybdenum for nitrogenase to work, as an integral element of the FeMo cofactor. This metal has to be delivered from the host plant to the bacteroids in order to synthesize the cofactor. However very little is known about the transporters mediating molybdenum supply to nodules of legumes in connection to SNF. MOT1 family has been found to transport molybdate in plants (1), what maked them good candidates to be involved in molybdenum delivery to nitrogen-fixing bacteroids.

We have identified a *Medicago truncatula* MOT1 member (MtMOT1.3) as responsible for molybdenum transport into rhizobia-infected cells. Heterologous expression in *Saccharomyces cerevisiae* shows that MtMOT1.3 is able to transport molybdate toward the cytosol. Its coding gene is mainly expressed in *M. truncatula* nodules, being 10-fold higher than the expression showed in roots or shoots. Immunolocalization studies show that MtMOT1.3 is located in the plasma membrane of infected and non-infected nodule cells. A loss of function *mot1.3* mutant exhibited reduced growth under symbiotic conditions, associated with a decreased nitrogenase activity compared to wild-type plants. This phenotype was rescued by the addition of molybdate to the nutritive solution or by genetic complementation with a wild-type *MtMOT1.3* copy. These results point to a role of MtMOT1.3 in molybdenum supply nodule cells, being a first step to understand Mo homeostasis in the legume-rhizobium symbiosis.

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POSTER 5B-17

NIT1, a Novel Component is Essential for Nitrate Inhibition of Nodulation in *Medicago truncatula*

Jie-Shun Lin and Fang Xie

National Key Laboratory of Plant Molecular Genetics, CAS Center for Excellence in Molecular Plant Sciences, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

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Legumes develop new root organ-nodule to fix nitrogen by successful symbiotic interaction with rhizobia. Rhizobia fix nitrogen in the nodule by using the plant provided photosynthesis products. Nitrogen fixation is an energy-consuming process and nodule number strictly controlled by plant through autoregulation of nodulation (AON) and environment nitrogen compounds levels. The molecular mechanism of AON pathway is quite clear, but the mechanisms of how high levels of nitrate inhibit nodulation still poorly understood (1). Previous researches revealed that AON pathway were involved in nitrate inhibition of nodulation, and CLE-RS2 was induced by nitrate (2-6). However, none of these mutants can form functional pink nodule when high level nitrate was available. In our study, two individual mutants of NIT1 (Nitrate Tolerance for nodulation 1) could produce mature pink nodule under high nitrate concentrations, while wild type only can form few bumps under the same condition. Graft assay suggested that nitrate tolerant of NIT1 was controlled by root, but not shoot. To further explore the function between NIT1 and AON during nitrate inhibition of nodulation, nitrate tolerance ability of *sunn-1* and *cra2-2* were assayed. The result showed that *cra2-2* was much sensitive to nitrate, *sunn-1* exhibited high nitrate tolerance, but could not form pink nodule. Moreover, graft and genetics assay suggested that CRA2 is essential for NIT1 nitrate tolerance ability, while SUNN play an important role in nodule number determination, but only have a weak role in nitrate inhibition of nodulation.

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POSTER 5B-18

Nitrogen Fixation by Faba Bean (*Vicia faba* L.) in a 4 Year Crop Rotation in East Scotland

Euan K. James, Laura Lopez del Egido, Marta Maluk, Mark Young, Cathy Hawes, Geoff Squire, Pietro P.M. Iannetta

The James Hutton Institute, Invergowrie, Dundee DD2 5DA, United Kingdom

Faba bean (*Vicia faba* L.) is the most important grain legume in the UK, being used for human consumption, as well as for livestock and fish feed. It forms an N-fixing symbiosis with *Rhizobium leguminosarum* sv. *viciae*, and is capable of obtaining all its N requirements through biological N-fixation (BNF). However, almost nothing is known about its ability to fix N in the UK, particularly for modern genotypes. To address this, grain yield, the proportion of nitrogen (N) derived from air (%Ndfa), total BNF, and the molecular diversity of nodule-associated *R. leguminosarum* (Mutch & Young, 2004) of five faba bean varieties were quantified in response to 'conventional' and 'sustainable' growing regimes in an experimental rotation at the Centre for Sustainable Cropping (CSC; www.hutton.ac.uk/csc) over four growing seasons (2012-2015). The method used for measuring BNF was the ^{15}N natural abundance method ((Unkovich et al., 2008), using non-legume weeds as references and B-values generated for each faba bean variety.

The beans obtained >90% of their N through BNF regardless of genotype, treatment and year, but the total amount of N fixed was dependent on grain and dry matter yield. Yield varied from year to year, with grain yield being 4 – 8 t Ha^{-1}, $year^{-1}$, depending on growing conditions. In a good year (e.g. 2012, 2014), faba bean fixed >200 kg Ha^{-1}, $year^{-1}$, but in a poor year (2013) this was halved. The residual N left in the field after grain harvest was >50 Ha^{-1}, $year^{-1}$, and if properly managed over winter this N can be made available to the next season's non-legume crop.

Nodulating isolates obtained from root nodules were distinguished by ribosomal 16S subunit (16S rRNA) PCR, and their genetic diversity assessed using sequencing of the symbiotic loci *nodA* and *nodD*. Rhizobia from nodules of cropped faba bean were similar from year to year, regardless of plant genotype and treatment, and could be grouped into two broad *nodD* clades, one containing only *V. faba* strains and the other containing a mixture of isolates from *V. faba* and from wild species of *Vicia* and *Lathyrus* that were growing adjacent to the experimental rotation.

It is concluded that modern faba bean varieties can obtain all their N-requirements through BNF in association with local indigenous rhizobial strains if managed properly, thus making substantial savings in terms of costly and environmentally damaging fertiliser applications.

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POSTER 5B-19

Study of Small RNAs and Their Related Synthesis Pathways in the Development of Nitrogen Fixing Nodules in the Model Legume *Medicago truncatula*

Jérémy Moreau[1], Vladimir Daric[1], Carine Satge[3], Hélène Proust[2], Céline Sorin[2], Lisa Tordjmann[2], Caroline Hartmann[2], Pascal Gamas[3], Martin Crespi[1], Christine Lelandais-Brière

[1] *Institute of Plant Sciences Paris Saclay IPS2, CNRS, INRA, Université Paris-Sud, Université Evry, Université Paris-Saclay, Orsay, France*
[2] *Institute of Plant Sciences Paris-Saclay IPS2, Paris Diderot, Sorbonne Paris-Cité, Orsay, France*
[3] *Laboratoire des Interactions Plantes-Microorganismes (LIPM), UMR INRA-CNRS 441/2594, Castanet Tolosan, France*

Small RNAs are essential regulators of gene expression during plant development, responses to the environment and plant microbe interactions. These non-coding RNAs, 21-24 nucleotides long, negatively regulate the expression of target genes at the post-transcriptional (PTGS) or transcriptional (TGS) levels. Since 10 years, around twenty different microRNAs, mainly targeting transcription factors, have been functionally associated to nodule development (reviewed in Lelandais-Brière et al., 2016). However, less is known about the roles of siRNAs in that process.

To analyze the dynamic and diversity of small RNA populations during root endosymbiosis in the model legume ***Medicago truncatula***, we previously performed high throughput sequencing of small RNAs from nitrogen-fixing nodules, roots inoculated with ***Sinorhizobium meliloti*** or treated with Nod factors (Formey et al., 2014; Lelandais-Brière et al., 2009). This allowed us to identify large sets of differentially expressed miRNAs, including legume-specific ones. Here we will present results of our recent analyses of miRNAs and siRNA populations in developing nodules (from 0 to 10 days after bacterial inoculation).

Small RNAs are produced after cleavage of long double-stranded or hairpin RNA precursors by enzymes of the RNase III family, called DICER-LIKE proteins (DCL). However, other RNase III encoding genes are present in the genome of ***M. truncatula***, whose roles in sRNA biogenesis remains to be determined. In addition, RNA-dependent RNA polymerases (RDR) are required for the biogenesis of certain siRNAs. For instance, RDR6 is involved in the production of secondary phased siRNAs involved in the PTGS of several disease resistance genes (Bustos-Sanmamed et al., 2014), while RDR2 is needed for the synthesis of heterochromatic siRNAs, which are involved in epigenetic regulations (Borges and Martienssen et al., 2015). Expression and functional analyses of different components of these siRNA pathways, including a novel nodule-specific atypical RNase III will be presented here.

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POSTER 5B-20

The Identification of the SST1 (Symbiotic Sulfate Transporter) Gene in *Medicago truncatula*

Gyöngyi Kováts[1], Á. Domonkos[1], B. Horváth[1], G. Iski[1], F. Ayaydin[2], P. Frendo[3], P. Kaló[1]

[1] *Agricultural Biotechnology Center, Gödöllő, Hungary*
[2] *Cellular Imaging Laboratory, Biological Research Center, Szeged, Hungary*
[3] *Sophia Antipolis Institute, INRA, Nice Sophia Antipolis University, CNRS, Sophia Antipolis, France*

Legumes are able to form symbiotic nitrogen-fixing interaction with rhizobia inducing the formation of a new organ called root nodule wherein nitrogen fixation takes place. In order to dissect the symbiotic interaction between *Medicago truncatula* and its compatible symbiotic partners, *Sinorhizobium sp.*, we analysed the alleles (5L and 11S) of an ineffective symbiotic (Fix-) mutant identified in a previous mutant screen. Both mutants showed the symptoms of nitrogen deficiency under symbiotic conditions and the microscopic analysis of the mutant nodules found colonized cells in the narrow invasion zone but no bacterial occupancy could be detected in the fixation zone. Sometimes we observed brown pigmentation in the fixation zone that might indicate defense responses induced by the deficiency of the symbiotic interaction. The analysis of the kinetics of the symbiotic phenotype revealed that the nitrogen-fixing interaction is blocked around 8 days post inoculation in the mutants. The map-based cloning approach identified the region of the *M. truncatula* genome where the homolog of the formerly identified symbiosis-specific sulfate transporter (*SST1*, Krussel et al., 2005) is located. The sequence analysis of the two mutant alleles revealed a 40 kbp deletion in the *sst1-2* genome affecting *SST1* and a 9 bp deletion in the *SST1* gene in *sst1-1*. We carried out complementation test to verify the identity of *MtSST1* and constructs encoding the fluorescent tagged SST1 was introduced into *M. truncatula* roots using *Agrobacterium rhizogenes* mediated transformation to analyze the sub-cellular localization of SST1.

Based on sequence similarity, SST1 is predicted to transport sulfate in nodule cells but this transportation has not been proved due the technical difficulties. To test that SST1 functions as a sulfate transporter, indirect analysis has been initiated and we measured the amount of sulfur containing compounds, such as cysteine and glutathione at different time-point following inoculation with rhizobia.

Nitric oxide (NO) is a signaling molecule in several developmental processes and it is involved in pathogenic and symbiotic plant-microbe interactions. In order to analyze the defense response that might be induced in *sst* mutants we measured the NO content of nodules developed on the roots of *sst* and other Fix- mutants.

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[1] *Krussel et al., 2005, Plant Cell, Vol. 17, 1625–1636*

POSTER 5B-21

The Role of Phased siRNAs in *Lotus japonicus* Development and Nodulation Symbiosis

Nikolaj B. Abel[1], Yaping Chen[1], Ana de Luis[2], Dennis B. Holt[1], Jens Stougaard[1] and Katharina Markmann[1]

[1] *Centre for Carbohydrate Recognition and Signalling (CARB), Department of Molecular Biology and Genetics, Aarhus University, Denmark*
[2] *Department of Experimental Sciences and Mathematics, Universidad Católica De Valencia, Spain*

Legumes such as pea, beans and the model trefoil *Lotus japonicus* develop nitrogen fixing root nodules in the presence of compatible rhizobial bacteria. Phased small interfering RNAs (phasiRNA) play key roles in the regulation of plant developmental processes including legume nodulation symbiosis. One group of phasiRNAs, the *TAS3* derived phasiRNAs (tasiR-ARFs), has been characterized in *L. japonicus* and shown to play a role in plant and symbiosis development (1, 2). In this study we dissect the phasiRNA pathway to verify *SUPPRESSOR OF GENE SILENCING 3* (*SGS3*), *RNA-DEPENDENT RNA POLYMERASE 6* (*RDR6*) and *DICER-LIKE4* (*DCL4*) to be required components of the phasiRNA generation pathway in *L. japonicus*. Our results suggest a role of *AGRONAUTE 7 (AGO7)* in processing tasiR-ARFs, but phasiRNAs stemming from other loci were *AGO7* independent. These loci, encoding two *NUCLEOTIDE-BINDING SITE LEUCINE-RICH REPEAT* (*NBS-LRR*) resistance genes and one *PENTATRICOPEPTIDE REPEAT* (*PPR*) gene, were sources of *SGS3, RDR6* and, partially, *DCL4* dependent phasiRNAs and were regulated by these in *cis*.

We show that *L. japonicus DCL4, AGO7, SGS3* and *RDR6* are required for quantitative regulation of infection and nodulation to similar levels, but qualitative development of both infection threads and nodules was normal in their absence. In *dcl4* mutants, quantitative impairment of symbiosis was observed despite the presence of residual populations of both tasiR-ARFs and other phasiRNAs, which we hypothesise are generated by other DCL enzymes. The intermediate tasiR-ARF populations observed in *dcl4* mutants seem active in silencing their regular targets, as indicated by the fact that *dcl4* mutants showed wild type like leaflet widths in contrast to *ago7, sgs3* and *rdr6,* which all displayed narrow, needle shaped leaflets.

Despite the presence of tasiR-ARFs also in belowground tissues in *dcl4* mutants we do not observe a similar rescue of symbiosis impairment compared to *ago7*, *sgs3* and *rdr6* mutants where tasiR-ARFs are absent. Our combined results thus suggest that apart from AGO7 dependent tasiR-ARFs, other phasiRNAs also contribute to symbiosis development.

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[2] *Li et al. (2014) New Phytologist 201: 531–544*

POSTER 5B-22

Tracing the Sulfur-Proteome of Nitrogen-Fixing Root Nodules in *Lotus japonicus*

Sebastian Schneider and Stefanie Wienkoop

Department of Ecogenomics and Systems Biology, University of Vienna, Austria

Lotus japonicus establishes nitrogen-fixing nodules in symbiosis with *Mesorhizobium loti*. The root nodules harbor the bacteroids that are surrounded by a peribacteroid membrane (PBM) formed from the plant plasma membrane. The PBM plays a central role in the metabolic exchange between the organisms. Besides nitrogen and sugars, the sulfur metabolism seems to play an important role for the functioning of the nitrogen fixation process (1). Several studies support this: (2) the sulfate transporter was found one of the most abundant PBM proteins; (3) evidence for a regulatory role of sulfate during drought acclimation and ethylene biosynthesis in nodules was presented as well as (4) the nodule function as important source of cysteine for the whole plant. However, the role of sulfur during nodule protein-synthesis and –functional regulation remains unclear.

Using a high throughput mass spectrometry based proteomics approach, we initially detected 1031 sulfur (cysteine or methionine) containing nodule proteins corresponding to 2685 different peptides. From those, 923 where plant and 1204 bacteroid specific. Compared to the root proteome, this resulted in a 2.3-fold higher number of identified nodule sulfur-containing proteins.

Focussing on those proteins, functionally annotated to be involved in sulfur metabolism, such as several plant and bacteroid specific cysteine synthase isoforms, we found altogether a number of 42 (compared to 16 proteins in roots). These data underpin the important role of sulfate as a key metabolic process in root nodules.

We then analysed the incorporation of reduced sulfur into the nodule proteome (plant and bacteroids) by using ^{34}S-metabolic labelling and mass spectrometry. Samples where taken 24, 48 and 72 hours after pulse labelling. Sulfur turnover dynamics are beeing presented and the role of sulfur in the context of nodule function and protein synthesis discussed.

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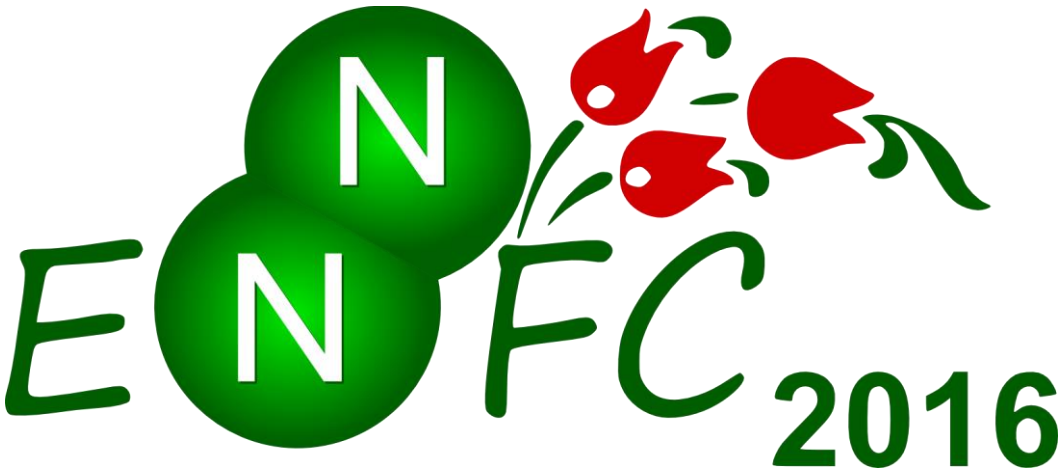

POSTER SESSION 6

Biological Nitrogen Fixation in Non-Legume Environments

Room Helia

POSTER 6-1 /LIGHTNING TALK/

The RNA Chaperone Hfq is a Global Regulator in the Nitrogen-Fixing *Pseudomonas stutzeri* A1501

Wei Wang[1], Yuhua Zhan[1], Wei Lu[1], Claudine Elmerich[2], Yongliang Yan[1], Min Lin[1]

[1] *Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China*
[2] *Institut Pasteur, Paris, France*

The nitrogen-fixing *P. stutzeri* A1501, isolated from the rice rhizosphere in China, fixes nitrogen due to the acquisition of a 49-kb nitrogen fixing island (1). Transcriptome analysis under nitrogen and oxygen limitation, or after ammonia shock, revealed dramatic changes in expression patterns of a number of genes related to carbon and nitrogen metabolism, located both in the *nif*-island and in the core genome, suggesting complex regulatory networks (2, 3).

The RNA chaperone Hfq was reported to be involved in many intracellular metabolic processes by promoting the interaction between protein or non-coding RNA and target mRNA, regulating the stability of mRNAs (4). An *hfq* gene was identified in the A1501 genome. Disruption of *hfq* did not affect the growth of *P. stutzeri* A1501 in rich or minimal medium. The *hfq* mutant strain was found to be impaired in several phenotypes including decrease resistance to oxidative and osmotic stresses, increased biofilm formation ability and decreased denitrification ability when nitrite was used as the electron acceptor. Real-time RT-PCR revealed significant decreased expression of the catalase *katA/B/E/G*, alkyl hydroperoxide reductase *ahpF/C* and glutathione peroxidase encoding genes, as well as decreased expression of the *nirB*, *nirS* encoding the key cytochrome C-552, cytochrome *cd1* nitrite reductase precursor and *nosR* encoding NosR regulatory protein.

We also found that *hfq* was involved in the regulation of nitrogen fixation, as its inactivation caused a significant decrease in nitrogenase activity. qRT-PCR revealed that expression of *nifA*, *nifK*, *glnA*, *glnK*, *rpoN* and *ntrC* were significant downregulated in the *hfq* mutant. Furthermore, Western blot analysis revealed a decreased amount of NifD and NifK polypeptides.

The above results showed that Hfq is involved in various physiological processes in *P. stutzeri* A1501. Further research will focus on the molecular mechanism of Hfq in regulation the expression of specific target genes.

References:

[1] *Yan et al. (2008) PNAS 105: 7564-7569*
[2] *Yan et al. (2010) BMC Genomics 11:11*
[3] *Dou et al. (2008) Chin Sci Bull 53 : 1197-1204*
[4] *Soper et al. (2010) Proc Natl Acad Sci USA 107 :9602-9607*

POSTER 6-2 /LIGHTNING TALK/

Identification and Functional Characterization of Genes Involved in Carbon Source Utilization in *A. brasilense* Sp7

Vijay Shankar Singh, Ashutosh Prakash Dubey, Ankush Gupta, Sudhir Singh and Anil Kumar Tripathi

School of Biotechnology, Banaras Hindu University, Varanasi, India

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Azospirillum brasilense is nitrogen fixing, non-photosynthetic, plant growth promoting ά-proteobacteria found in the close vicinity of various plant roots (1) including C3 cereals and C4 grasses. Organic acids (mainly C4-dicarboxylates) as well as trace amount of sugars and sugar alcohols of root exudates act as preferred source of carbon and energy for the nitrogen fixation and for the growth (2). To search the genes and proteins involved in C4-dicarboxylates utilization, 2D gels of *A. brasilense* grown in different C4-dicarboxylates supplemented medium have been resolved and observed that a DctP protein is upregulated in malate grown culture while nearly constant expression has been observed in succinate and fumarate grown cultures of *A. brasilense*. Insertional inactivation of induced *dctP* and double knock-out mutant of *dctP* and another C4-dicarboxylates transporter *dctA* have shown that DctP is a major transporter (~75% growth retardation in *dctP::km* mutant) while DctA (~25% growth retardation in *dctA::gm* mutant) is a minor C4-dicarboxylates transporter in *A. brasilense*. Enhanced promoter activity of *dctP* and *dctA* at µM and mM range of substrates, respectively have been shown that DctP is high affinity while DctA is low affinity transporter and enhanced promoter activity of *dctP* in *σ54::km* while zero activity of *dctA* promoter have been shown that σ54 positively regulate the expression of *dctA* gene. In addition to C4-dicarboxylate transporters we observed a PQQ dependent quinoprotein alcohol dehydrogenase (ExaA) protein has been upregulated in glycerol as well as fructose grown cultures of *A. brasilense* and belongs to Type-I of PQQ-ADH. 5' RACE study predicted the σ54 binding site and it has been demonstrated that σ54 regulate positively and another RpoH2 sigma factor regulate negatively the expression of *exaA* gene. Role of divergently organized two component system as well as regulator binding site have been predicted by mutations and it has been shown that LuxR type of regulator EraR regulate the expression of *exaA* by showing positive interaction with promoter bounded σ54-RNA Polymerase complex without having GAFTGA domain.

References:

[1] *Bashan et al. (1990) Can. J. Microbiol. 36:591-608.*
[2] *Del Gallo et al. (1994) Okon Y (ed) Azospirillum/plant associations. CRC, Boca Raton, Fla., pp 57-75.*

POSTER 6-3 /LIGHTNING TALK/

Development of Tools for Transformation and Gene Expression in *Paenibacillus* Species and Complete Genome Sequence of *Paenibacillus riograndensis* SBR5

Luciana Fernandes de Brito[1], Marta Irla[1], Tatjana Walter[1] and Volker F. Wendisch[1]

Chair of Genetics of Prokaryotes, Faculty of Biology & CeBiTec, Bielefeld University, Bielefeld, Germany

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Paenibacillus riograndensis is a rod-shaped, Gram-positive, nitrogen-fixing bacterium. The strain SBR5 was isolated from the rhizosphere of wheat plants cultivated in Rio Grande do Sul, Brazil. In addition to nitrogen fixing, SBR5 possesses further plant growth promoting activities, e.g. production of indol-3-acetic acid and siderophores. Thus, *P. riograndensis* SBR5 is interesting for agricultural purposes, although it has not been studied much.

Here, we have determined the complete genome sequence of SBR5 and completely annotated the genome. The genome consists of one chromosome with 7.893.056 bps, containing 6705 protein coding genes, 87 tRNAs and 27 rRNAs (1).

We also developed a new transformation protocol for *Paenibacillus* species based on physical permeation through mixing the cell suspension with a plasmid-aminoclay solution (2). Transformation was shown by plasmid isolation and re-transformation as well as by heterologous production of a fluorescent reporter protein. Furthermore, the *gfpUV* reporter gene was used to test rolling-circle and theta-replicating plasmids for constitutive and inducible expression. Fluorescence activated cell scanning (FACS) verified the versatility of the developed expression vectors for constitutive and graded inducible expression. These gene expression systems could be transferred to another Paenibacillus species, i.e. *P. polymyxa*. In addition, inducible gene expression was applied to metabolic engineering of *P. riograndensis*.

References:

[1] *Brito et. al. (2015) J. Biotechnol. 207: 30–31.*
[2] *Choi et. al. (2013) J. Microbiol. Meth. 95: 97–101.*

POSTER 6-4 /LIGHTNING TALK/

The Regulation of Nitrogen Fixation and Assimilation in the Associative Diazotroph *Klebsiella oxytoca* M5a1

Christopher J. Waite, V. Elsin, R. Berchtold, T. Tanny, J. Gan, N. Sanguankiattichai, S. Amartay, M. Buck, J. Schumacher

Division of Cell and Molecular Biology, Department of Life Sciences, Imperial College Road, Imperial College, London, United Kingdom

Engineering free-living and associative diazotrophic bacteria for the release of fixed nitrogen (N) into the rhizosphere represents one promising strategy for meeting the global demand for agricultural fertiliser sustainably (1-2). As part of an ongoing collaborative project (BB/N003608/1) aiming to rationally re-engineer the cell signalling and metabolism of model diazotrophs for the supply of surplus reduced N (e.g. ammonium) to plants, we present a preliminary model for the regulatory interplay between N fixation and N assimilation in the associative, soil-dwelling bacterium *Klebsiella oxytoca* M5a1 (*Ko*). In most bacteria the rate of N assimilation is coupled to the cellular N status (glutamine/α-ketoglutarate ratio) by a regulatory cascade involving post-translational uridylylation of PII type proteins (GlnB and GlnK) and the σ^{54}-type transcriptional activator NtrC (3). In diazatrophs such as *Ko*, these proteins also regulate the expression of the *nif* gene cluster, encoding the nitrogenase complex and its associated factors, via a second, downstream σ^{54}-type transcriptional activator NifA (4). Nitrogenase expression is coupled tightly to internal N status (via NtrC), anaerobiosis (via NifL, the negative regulator of NifA) and import of exogenous fixed N (via interactions between GlnK and the primary ammonium transporter, AmtB (5)). Ultimately, the co-regulation of the *gln* (N assimilation) and *nif* (N fixation) regulons by multifunctional regulatory proteins affords highly economical nitrogen metabolism, according to supply and demand, in which a surplus of fixed N compounds is minimised.

Ko provides a suitably characterised model diazotroph in which to develop an integrated systems-level understanding of N economy management, including identification of key nodes of control and robustness, whether catalytic or regulatory, that prevent excess fixed N production and export. In preparation for omics analyses (RNA-seq, targeted MRM-MS proteomics and LC-MS metabolite profiling) we have characterised multiple key parameters during the diazatrophy transition that follows ammonium run-out including (a) cell growth rate, (b) transcription of key N regulons, (c) nitrogenase activity and (d) critical O_2 concentrations. We have developed an O_2-independent fluorescent gene reporter system and a library of regulatory mutants (both gene deletions and CRISPR-targeted substitutions) which together reveal novel control mechanisms at play in this organism. We report initial findings employing synthetic transcription factors, for instance a chimera of the O_2-sensitive *Bradyrhizobium japonicum* and native *Ko* NifA homolgoues, to redirect/tune *nif* gene expression and thereby uncouple the N demand of the cell from N fixation activity.

References:

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[2] *Setten et al. (2013) PLoS One, 8:e63666.*
[3] *Schumacher et. al. (2013) mBio. 4:e00881-13.*
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[5] *Gruswitz et al. (2007) PNAS. 104:42–7.*

POSTER 6-5 /LIGHTNING TALK/

Engineering a Biased Plant Rhizosphere to Establish Synthetic Symbioses in Cereals

Ponraj Paramasivan[1], Barney A. Geddes[2], Philip Poole[2] and Giles Oldroyd[1]

[1] *Department of Cell and Developmental Biology, John Innes Centre, Norwich, United Kingdom*
[2] *Department of Plant Sciences, University of Oxford, Oxford, United Kingdom*
E-mail: ponraj.paramasivan@jic.ac.uk

One strategy to engineer plant-microbe interactions in the rhizosphere to enhance crop production in future agroecosytems is to create biased rhizospheres that benefit the plant. A well-known natural instance of a biased rhizosphere is the opine concept during pathogenic *Agrobacterium* - plant interactions. In this example plant production of opines benefit the *Agrobacterium* strains that carry the genes for opine catabolism. Since engineering opine synthesis into the rhizosphere will enrich phytopathogenic agrobacteria, an alternative plant-produced carbon substrate to engineer a biased rhizosphere is rhizopine. Rhizopines are nodule specific, opine-like compounds synthesized by a few rhizobial strains in root nodules and exuded into the rhizosphere. Rhizopine give plant-nodulating rhizobia a nutritional advantage in the rhizosphere over other saprophytic microorganisms. Current advances in plant and microbial synthetic biology will enable us to use metabolic engineering to create a rhizopine-based biased rhizosphere and establish synthetic symbioses for enhanced nitrogen provision to cereals.

We aim to engineer LCO inducible production of rhizopines as a novel substrate in cereal roots (barley) that can specifically support nitrogen fixation by genetically modified, rhizopine catabolising bacteria which carry the ability to fix some level of nitrogen in the free-living state. In this context we are analysing the constitutive and inducible (Nod factor) production of rhizopines in transient plant transformation systems so that this approach can be transferred to cereals.

This work is supported by UK-BBSRC and US-NSF joint funding.

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[2] *Geddes et. al. (2014) Curr. Opin. Biotechnol. 32: 216-222.*

POSTER 6-6 /LIGHTNING TALK/

Signaling Pathway in the Actinorhizal Root Nodule Symbiosis

Sergio Svistoonoff[1,6], Valérie Hocher[1], Leandro Imanishi[2], Laurent Brottier[1], Elodie Pirolles[1], Daniel Moukouanga[3], Mireille Chabaud[4], Joëlle Fournier[4], David Barker[4], Luis Wall[2], Louis Tisa[6], Didier Bogusz[3], Claudine Franche[3], Hassen Gherbi[1]

[1] *IRD-LSTM, Campus de Baillarguet, Montpellier, France*
[2] *Laboratorio de Bioquímica, Microbiología e Interacciones Biológicas en el Suelo, Universidad Nacional de Quilmes R. Sáenz Peña 352, Bernal, Argentina*
[3] *IRD, UMR DIADE, Campus Agropolis, Montpellier, France*
[4] *LIPM, INRA (UMR 441), CNRS (UMR 2594), Castanet-Tolosan, France*
[5] *Department of Molecular, Cellular and Biomedical Sciences, University of New Hampshire, Durham, United States*
[6] *Laboratoire Commun de Microbiologie IRD/ISRA/UCAD, Sénégal*

Legumes and a phylogenetically diverse group of species called actinorhizal plants are able to establish N_2-fixing Root Nodule Symbiosis (RNS) in association with rhizobia and *Frankia* bacteria, respectively. Legumes and actinorhizal plants all belong to the Fabid Clade suggesting that the ancestor of extant Fabids acquired a genetic predisposition towards nodulation. However the genetic basis of this predisposition is still unknown. In model legumes, perception of rhizobial signals (Nod factors) by LysM-RLKs receptors activates a signaling cascade that then leads to the formation of root nodules. Part of this pathway is also essential for the arbuscular mycorrhizal (AM) symbiosis and is referred to as the Common Symbiotic Signaling Pathway (CSSP).

We are investigating the genetic bases of symbiotic signaling in actinorhizal symbioses. Using transcriptomic and functional studies, we discovered that many genes involved in the CSSP required for nodulation and mycorrhiza formation in legumes are also present in actinorhizal species and showed that at least two of these, *SymRK* and *CCaMK*, are essential for the establishment of actinorhizal symbiosis (1). In addition, we demonstrated that *NIN* gene, a transcription factor required for rhizobium-legume symbiosis is also essential for nodulation in the actinorhizal species *Casuarina glauca,* thus unveiling the first element of a common nodulation-specific pathway (2). Furthermore, by developing sensitive bioassays, we revealed that cell-free culture supernatants of *Frankia* CcI3 strain are able to induce both sustained high frequency Ca^{2+} spiking and transcriptional activation of Pro*CgNIN:GFP* in root hairs of *C. glauca*. Based on these two bioassays, we showed that biological active fraction present in *Frankia* CcI3 supernatant is hydrophilic, of low molecular weight and resistant to chitinase degradation (3).

Altogether, these studies provide new insights both into the molecular dialogue between *Frankia* and actinorhizal species, and into the evolution of RNS.

References:

[1] *Svistoonoff et al. (2014) Curr. Opin. Plant Biol. 20: 11-18.*
[2] *Clavijo et al. (2015) New Phytol. 208(3):887-903.*
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POSTER 6-7 /LIGHTNING TALK/

Functional Genomics of Cyanobacteria in Symbiosis with Boreal Feather Mosses

Denis Warshan[1], Christopher L. Dupont[2], Philip D. Weyman[3], Ulla Rasmussen[1]

[1] *Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, Sweden*
[2] *Microbial and Environmental Genomics Group, J. Craig Venter Institute, La Jolla, United States*
[3] *Synthetic Biology and Bioenergy Group, J. Craig Venter Institute, La Jolla, United States*

The nitrogen cycling in boreal forest ecosystems is largely determined by a symbiotic association between feather mosses (*Pleurozium schreberi* and *Hylocomium splendens*) and diazotrophic cyanobacteria that fix majority of nitrogen flowing into boreal ecosystems (1). Because nitrogen is often limiting in boreal forests, the interaction between the cyanobacteria and the mosses greatly affects the productivity of this ecosystem that makes up almost 30% of Earth's forested land (2-3). We seek to understand the genetic diversity of the cyanobacteria associated with the mosses and the molecular steps leading to the moss-cyanobacterial symbiosis.

We sequenced the genomes of five different *Nostoc* spp. that are able to form symbiotic associations with feather moss. As a control, we also sequenced the genome of one *Nostoc* sp. that is unable to form symbioses with the mosses. Comparative genome analysis of these cyanobacterial species allowed us to probe the genomic diversity of moss-associated *Nostoc* strains and identify a set of 32 genes differentially retained in genomes of symbiotic competent cyanobacteria compared to the non-symbiotic competent strain.

We also obtained transcriptomic and proteomic data for *Nostoc* grown in isolation, together, or with chemical contact with the moss only through filter separation. Transcriptomic and proteomic data revealed that differentially retained genes and their neighborhood are upregulated in chemical contact (gas vesicles, chemotaxis related genes), during both condition (pyrroloquilonine-quinone and exopolysaccharide production) and together with the moss partner (taurine catabolism and aliphatic sulfonate transporter). Thus, we hypothesize that cyanobacteria symbiotic gene clusters are essential to establish the symbiosis with feather moss.

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POSTER 6-8 /LIGHTNING TALK/

Evolution of the Actinorhizal Symbiosis: Analysis of Bacterial Genomes of the Basal Cluster

Than Van Nguyen and K. Pawlowski

Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, Sweden

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The ability to establish root nodule symbioses is restricted to four different plant orders, the Fabales, Fagales, Cucurbitales and Rosales which together form the fabid clade. With the exception of *Parasponia* sp., all host plants of rhizobia symbiosis belong to the order Fabales. The other three orders contain a diverse group of plants within eight different families that enter into nitrogen-fixing symbiosis with soil actinobacteria of the genus *Frankia.* Phylogenetically, symbiotic *Frankia* strains can be divided into three main clusters. Members of cluster II nodulate the broadest range of host plants with species from four families from two different orders, growing on six continents. This cluster also forms the basal group of the genus *Frankia.*

Of the three genomes available from cluster II, two originated in Asia - Dg1 from Pakistan (1), BMG5.1 from Japan (2) – and one from North America – Dg2 from California, USA (unpublished). Based on average nucleotide identity analysis, BMG5.1 and Dg1 represent the same species, while Dg2 represents another species. This indicates that there is less species diversity in cluster II than in clusters I or III. Cluster II strains contain features not present in the other two clusters, e.g., Dg1 and Dg2 (but not BMG5.1) contain the canonical *nod* genes *nodABC* which are expressed in nodules. All cluster II strains contain several copies of the mammalian cell entry (*mce*) genes which encode steroid transporters in other actinobacteria. These and other features of cluster II genomes will be discussed.

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POSTER 6-9

An Ethanol Responsive Hierarchical Signal Cascade - Important for the Endophytic Life of *Azoarcus* sp. BH72

Andrea Krause, Theresa Harten, Henrike Julich, Manasee Mankar, Barbara Reinhold-Hurek

Department of Microbe-Plant Interactions, University of Bremen, Bremen, Germany

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The habitat of the nitrogen-fixing endophyte *Azoarcus* sp. BH72 is the root apoplast of grasses grown under waterlogged conditions. Rice cultivated under these conditions produces and accumulates ethanol. Since ethanol diffuses through plant membranes, it would appear in the apoplast and could serve there as sole carbon source for endophytes. To metabolise ethanol, strain BH72 is with eight alcohol dehydrogenases (ADH) well equipped (1). For three ADHs, expression was ethanol-inducible under aerobic and microaerobic conditions, of which two (ExaA2, ExaA3) were also expressed inside rice roots. Disruption of these genes reduced growth of *Azoarcus* on ethanol-containing media and diminished competitiveness during endophytic colonisation (2). Similarly, the aldehyde dehydrogenase AldA is important for growth of *Azoarcus* on ethanol – an enzyme encoded by a gene whose expression profile was also ethanol dependent and detectable inside of infected rice roots.

exaA2 and *exaA3* are clustered in the genome surrounded by genes encoding two two-component regulatory systems (TCS) termed ExaS-ExaR and ElmS-GacA. Functional genomics revealed (a) that expression of the corresponding genes was induced by ethanol under aerobic and microaerobic conditions, (b) that the genes were also expressed in close association with or even inside of rice roots, (c) that both TCSs were indispensable for growth on ethanol, and (d) that they were important for competitiveness during rice root colonisation. Both regulatory systems are forming a hierarchically organised ethanol responsive signal transduction cascade with ExaS-ExaR as highest level, essential for effective expression of the ethanol oxidation system based on ExaA2 and AldA. No influence of any TCS on the ethanol induced *exaA3* expression was detectable. In contrary, transcription and expression levels of *exaA3* increased when any of the *tcs* genes was deleted. An additional element of this regulatory signal cascade is RpoN. Disruption of *rpoN* inhibited *Azoarcus* to grow on ethanol and influenced the ethanol-induced expression of the ethanol oxidation system as well as of the *tcs* genes which coincides with a σ^{54}-dependent promoter prediction upstream of the corresponding genes.

All this together underlines the importance of ethanol for the endophytic lifestyle of *Azoarcus* sp. BH72 but indicates also a tight regulation of the ethanol oxidation system during root colonisation.

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POSTER 6-10

Diversity and Activity of Diazotrophs Associated with Micro-environments of Wetland Rice

Hannes Schmidt, Dagmar Woebken

Department of Microbiology and Ecosystem Science, Division of Microbial Ecology/University of Vienna, Vienna, Austria

Rice is one of the world's most important crop plants. The production is strongly limited by nitrogen (N), which is typically supplied by industrial fertilizers that are costly and hazardous to the environment. It is known that *Biological Nitrogen Fixation* through N_2-fixing bacteria and archaea (diazotrophs) can alleviate the N-shortage in rice cultivation. However, our knowledge on the micro-sites of N_2 fixation, as well as the diversity and *in situ* N_2 fixation activity of diazotrophs in the soil-microbe-plant interface (i.e. rhizosphere) of flooded rice fields is still rudimentary.

Greenhouse studies were performed to identify key factors that control rice-diazotroph association and related N_2 fixation activities. Paddy soils of different geographical origin were cultivated with two agriculturally used genotypes of wetland rice. Samples were separated into bulk soil, rhizosphere soil, rhizoplane, and roots at flowering stage of rice plant development. These samples were subjected to functional assays and various molecular biological techniques to identify the inhabiting diazotroph community.

Based on Illumina amplicon sequencing of 16S rRNA and *nifH* genes and transcripts, we will present insights into the diversity and potential activity of bacterial/diazotroph communities associated with the different rice micro-environments. Emphasis will be put on comparatively discussing the influence of (a) the soil microbial "seed bank" and (b) plant genotype in shaping the microbiomes. Actual N_2 fixation activities of soil-genotype combinations and micro-environments will be shown on the basis of incubation assays using $^{15}N_2$-containing atmospheres. Areas of potential N-transfer between diazotrophs and rice roots will be presented via the detection and visualization of spatial colonization patterns of selected diazotrophic groups on rice rhizoplanes.

POSTER 6-11

Green Alder (*Alnus viridis*, Chaix, DC) Encroachment Shapes Differently Fungal and Bacterial Communities in Subalpine Soils

Guillaume Schwob[1], Mélanie Roy[2], Thomas Pommier[1], Maria P. Fernandez[1]

[1] *Laboratoire Ecologie Microbienne, UMR CNRS 5557 / UMR INRA 1418, Université Claude Bernard Lyon I, Villeurbanne, France*
[2] *Laboratoire Evolution et Diversité Biologique, UMR 5174, Toulouse, France*

Since the abandonment of human agropastoral activities in Alps mountains, subalpine grasslands undergo a rapid and progressive biological invasion of *Alnus viridis* shrubs. Thanks to its stolons, its nitrogen fixing symbiose with the actinobacteria *Frankia* and its ectomycorhizal cohorts, green alders outcompete others subalpine tree species and quickly evolve from a mosaic stage of some patchy individuals to a thick and closed forest associated with a loss of biodiversity (1).

While impacts of *Alnus viridis* encroachment on soil properties, plant, arthropod and ornithological diversities are well described (2,3,4), little is known about its density effect on soil microbial communities, its symbionts (bacterial and fungal) detection and distribution in soils and on the functional modifications in response to this ecological succession in subalpine ecosystems.

The 3 colonization stages (grassland, mosaic and forest) were studied in two different sites in the Vanoise National Park (Savoie, France). Symbionts distribution, microbial richness and communities structure in both soils and nodules were analyzed by metabarcoding of 3 bacterial (*16S rDNA*, *nifH* and *amoA*) and 1 fungal (ITS1) genes. Pedological analysis were performed and functional diversity of N cycle bacteria evaluated by nitrification and denitrification enzyme assays.

Frankia, and *Alnus*-specific ectomycorhizal fungi, were detected in all samples regardless of the ecological stage. While *Frankia* abundance and diversity didn't vary significantly, ectomycorhizal fungi abundance increased together with the increase of the host density. Site and colonization stage both shape fungal communities structure. In the case of bacteria, one site harboured ecological stage effect on both community structures and functional activities. Indeed, each colonization stage revealed specific ammonia oxidizing and N2 fixing communities and different nitrification activity levels.

These results show that (i) *Alnus viridis* drives differently bacterial and fungal communities, (ii) its encroachment impacts both structural and functional diversity of bacteria related to the N cycle, (iii) the distribution in soils of its bacterial symbionts (*Frankia*) is weakly influenced by the host density. These results are discussed in the light of the possible status of obligate symbiont of *Frankia* strains associated to green alder (5). This study provides a better understanding of the impact of *Alnus viridis* encroachment on abandoned subalpine pastures and the seek for optimal solutions of management.

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POSTER 6-12

Growth Promotion and Nitrogen Metabolism of Two Sugarcane Varieties Inoculated with Diazotrophs

Silvana G. dos Santos[1], Flaviane da S. Ribeiro[1], Camila S. da Fonseca[1], Willian Pereira[1], Leandro A. Santos[1] and Veronica M. Reis[2]

[1] *Universidade Federal Rural do Rio de Janeiro, Dept. de Solos, Seropédica, RJ, Brazil*
[2] *Embrapa Agrobiologia, Seropédica, RJ, Brazil*
veronica.massena@embrapa.br

The objective of this study was to evaluate the initial development and nitrogen metabolism (N) in seedlings of two sugarcane varieties inoculated with diazotrophs. An experiment was developed from three stages of cultivation: sprouting in box with sterile sand and vermiculite substrate, plastic tubes containing commercial substrate Multiplant® not sterile and hydroponics from modified Hoagland solutions. The treatments tested were a mixed inoculant consisting of five strains of diazotrophs: *Gluconacetobacter diazotrophicus* (Gd - strain BR11281^{T} - PAL-5^{T}) *Herbaspirillum seropedicae* (Hs - BR11335 - HRC54), *Herbaspirillum rubrisubalbicans* (Hr - BR11504 = HCC103), *Burkholderia tropica* (Bt - BR11366^{T} = PPe8^{T}) and *Azospirillum amazonense* (Aa - BR11145 = CBAMc), two sugarcane varieties, RB867515 and IACSP95-5000 and the hydroponics phase evaluated under two N rates high (3 mM) and low (0.3mM) and under restriction of nutrient (72 h without N). At the end of each repetition stage were aimed at different evaluations. After sprouting evaluated the shoot (SDM) and roots dry mass (RDM), volume and root area, root length by thickness class and number of bifurcations. In tubes phase evaluated the SDM and RDM, height and stem diameter and length and width of the leave^{+1}, while during and after the hydroponics were evaluated the activity of the nitrate reductase and glutamine synthetase enzymes, soluble fractions, dry biomass and nutrient content.

The RDM after sprouting increased by 50% by inoculation. This effect was also observed with the aid of WinRHIZO® Arabidopsi software in volume and root area. Moreover, inoculation increased the fine root length by approximately 30% in the variety RB867515. After cultivation in tubes inoculated seedlings of both varieties had higher RDM and SDM. In hydroponic cultivation it was observed that the inoculation increased the nitrate reductase activity (NRa) in leaves and roots and glutamine synthetase (GS) in the leaves. The effect of NRa was found only in the variety RB867515 and after N restriction treatment, while for GS there was no variety distinction. Inoculation increased the N-nitrate content in the leaves and reduced levels in roots. The inoculated plants also showed an increase in N-amino content in the roots, with the largest effects was observed on N restriction. At the end of hydroponics, plants of the variety RB867515 inoculated showed higher RDM, dry mass of secondary tillers, total dry matter and N content in the stem.

POSTER 6-13

Growth Promotion of Two Maize Hybrids by Inoculation with Different PGPR

Dragana Dj. Bjelic, Nastasija B. Mrkovacki, Jelena B. Marinkovic, Branislava B. Tintor

Institute of Field and Vegetable Crops, Novi Sad, Serbia

Plant growth promoting rhizobacteria (PGPR) enhance the plant growth directly by assisting in nutrient acquisition and modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens in the forms of biocontrol agents (1). Growth promotion and disease control by PGPR is a result of complex interrelated processes that include synthesis of phytohormones, phosphate solubilization, nitrogen fixation, production of siderophores, antibiotics, hydrogen cyanide, and various lytic enzymes (2). Many authors have shown that the growth–promoting ability of some rhizobacteria may be highly specific to certain plant species, cultivar and genotype and pointed to the possibility of selection of PGPR strains on plant genotype level (3). Therefore, the aim of this study was to examine the PGP properties of eight isolates and their effect on microbial community structure in rhizosphere, growth and N, P, Zn content of two maize hybrids grown in field conditions.

In search of efficient PGPR strains with multiple activities, 50 isolates were obtained from maize rhizospheric soil in Vojvodina Province. Isolates were biochemically characterized and screened *in vitro* for lytic enzyme production, plant growth promoting properties and antifungal activity (against *Helminthosporium* sp., *Macrophomina* sp., *Fusarium* sp.). The trial was established on calcareous chernozem soil at the Rimski Šančevi experimental field (45°20' latitude, 19°51' longitude, 86 m altitude) of Institute of Field and Vegetable Crops, Novi Sad, Serbia. Maize seeds were obtained from the hybrids NS 6010 and NS 6030, with a medium late vegetative cycle (FAO 600). Inoculation was performed with liquid culture (≈2-5 x 10^{10} CFU ml^{-1}) of isolates. Tests were conducted on three isolates of *Azotobacter* sp. (A5, A8, A13), two isolates of *Bacillus* sp. (B9, B16) and *Pseudomonas* sp. (P1, P5), one isolate of *Streptomyces* sp. (S6), as well as their mixture, while non-inoculated seeds were designed as control.

This study showed that most isolates of free-living rhizobacteria from maize rhizosphere exhibit PGP traits which can directly or indirectly promote plant growth. Inoculation increased the total number of microorganisms (33-66%), number of azotobacters (13-31%), N_2-fixing bacteria (35-100%) ammonifiers (10-61%), P-mobilizing bacteria (33-105%), actinomycetes (5-47%), and dehydrogenase activity (1-47%) in rhizosphere, as well as height (3-12%), dry weight (23-75%), N (12-20%), P (2-15%) and Zn (1-24%) content of maize plants. The best effect on microbial activity in the rhizosphere was achieved by co-inoculation, while better effect on growth and N, P, Zn content of maize hybrids was obtained by using individual inoculants. Different response of tested hybrids to inoculation was also reported, with a higher effect in the hybrid NS 6010.

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POSTER 6-14

Mechanistic Studies of Bacterial Plant Growth Promotion Using the Grass Model Plants *Brachypodium distachyon* and *Setaria viridis*

Fernanda Plucani do Amaral[1], Vania Carla S. Pankievicz[2], Ana Arisi[3], Emanuel M. de Souza[2], Fabio Pedrosa[2] and Gary Stacey[1]

[1] *Divisions of Plant Science and Biochemistry, C.S. Bond Life Science Center, University of Missouri, Columbia, Missouri, United States*
[2] *Department of Biochemistry and Molecular Biology, Federal University of Parana, Curitiba, PR, Brazil*
[3] *Department of Food Science and Technology, Federal University of Santa Catarina, Florianopolis, SC, Brazil*

Plants interact with a wide range of soil microorganisms. In some cases, this interaction can result in significant benefits to both microbe and plant host. It is well known that some soil bacteria can promote the growth of plants increasing crop yield. The effects of such 'plant growth promoting bacteria' (PGPB) have been well documented with a variety of plant species. One lesson from such studies is that both the bacterial and plant genotype are critical in order to induce significant growth promotion. However, the molecular mechanisms behind this growth promotion are still largely unclear. We believe that the adoption of a suitable model bacterial-plant system could significantly accelerate research to increase our mechanistic understanding of these important associations. Our previous studies demonstrated significant plant growth promotion in the model grass species *Setaria viridis* (1) and *Brachypodium distachyon* (2) in association with *Azospirillum brasilense*, *Herbaspirillum seropedicae* and/or *Azoarcus olearius*. We are now expanding on these initial studies to discover and characterize important bacterial and plant genes essential for the interaction, as well as for plant growth promotion. For example, we are using RNA-seq to identify genes induced within the host species in response to bacterial inoculation. The use of a plant model species provides the ability to further explore the function of these genes using transgenic and/or genetic methods. As a complement to these studies, we are also using TnSeq, a high-through-put method for transposon mutagenesis, to identify and then subsequently characterize Azoarcus genes essential for root colonization. The results of this experiment identified over 90 candidate genes whose presence appears to be essential to successful root association. Further investigation of the plant and bacterial genes identified through our studies should increase our mechanistic understanding of these associations. Our ultimate goal is to utilize this information to manipulate the plant-PGPB association to maximize the utility to agriculture, including the growth of bioenergy grass species on marginal land.

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POSTER 6-15

Modelling and Engineering *Anabaena* sp. PCC 7120 for Ammonia Excretion

David Malatinszky[1], Ralf Steuer[2], Dennis Nürnberg[1], John Rowland[1], Paulina Bartasun[1] and Patrik Jones[1]

[1] *Department of Life Sciences, Imperial College London, United Kingdom*
[2] *Institute for Theoretical Biology, Humboldt University Berlin, Germany*

Nitrogen is an essential element for every organism on Earth. Modern agricultural activity depletes soil in nitrogen much faster than it is naturally replenished. Therefore, fertilization is key for feeding a fast-growing population, spreading every year hundred-thousand tons of nitrogen on agricultural land worldwide (1). The use efficiency of fertilizer nitrogen is, however, relatively low, and the pollution caused costs the EU and other societies tens of billions of euros annually in environmental remediation costs (2). A more targeted application would be important by e.g. using nitrogen-fixing organisms in close association to agricultural crops. Among these organisms filamentous cyanobacteria form highly specialized heterocysts to protect nitrogenase from inactivation by oxygen (3). These cells provide neighbouring vegetative cells with assimilable nitrogen in return for a source of carbon and electrons. To excrete some of this fixed nitrogen we first had to understand the nature of metabolite exchange between vegetative cells and heterocysts. To this end, we created a two-cell genome-scale stoichiometric model for *Anabaena* sp. PCC 7120. The potential metabolite exchange between the two cell types was found to be highly flexible, with several metabolite sets providing higher growth rates than those metabolites suggested in literature. Using flux balance analysis of diazotrophically grown filaments we investigated the stoichiometric yield of nitrogen-containing compounds. Excretion of ammonia achieved the highest yield and metabolic engineering strategies to increase its intracellular pool were therefore designed. In *Anabaena* sp. PCC 7120, the glutamine synthetase GlnA is responsible for the rapid assimilation of ammonia produced by nitrogenase. The level of this protein is natively controlled by a small polypeptide, IF7A, which was therefore overexpressed. In another strain, *glnA* was replaced for an active-site mutant bearing lesser activity on ammonia. Furthermore, the *amt* cluster encoding three ammonium uptake transporters was knocked out in both strains to abolish recapture of ammonia lost via diffusion.

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POSTER 6-16

PGPR in Sugarcane When, Where and How? - Initial Colonization of Different Bacterial Strains Visualized *in situ* Combined with PGPR Related Transcript Quantification

Doreen Fischer[1], Andreas Hofmann[1], Luc. M. F. Rouws[1], Stefan Schwab[1], Anton Hartmann[2], José Ivo Baldani[1], Veronica M. Reis[1]

[1] *Embrapa Agrobiologia, BR 465 km 7, Seropédica, Rio de Janeiro, Brazil*
[2] *Research Unit Microbe-Plant Interactions, Helmholtz Zentrum München, German Research Center for Environmental Health, Neuherberg, Germany*

Sugarcane is one of the most important agricultural plants of the world, used as source for biofuels and renewable energy. Large amounts of chemical fertilizers are necessary to successfully grow sugarcane in many regions, so there is a dire necessity to increase the productivity using sustainable agricultural practices. Due to the evidence for nitrogen fixation of sugarcane associated bacteria, N-balance experiments as well as 15N-dilution measurement have been performed and showed significant contributions of biological nitrogen fixation in sugarcane (Urquiaga et al., 2012). Evidence accumulated already in the 1980s for diazotroph endophytic living microorganisms and novel bacteria like *Gluconacetobacter diazotrophicus, Herbaspirillum seropedica, Herbaspirillum rubrisubalbicans, Azospirillum amazonense* and *Burkholderia tropica* were isolated from the interior of sugarcane tissue and recently used in field applications to increase the yield (Schultz et. al, 2014). The role of each bacterial species in the high ability of BNF in some sugarcane varieties as well as the plant growth promoting effect by production of phytohormones remain unclear until now. A mixture of the five bacterial species is used as inoculum on field scale since years and lots of efforts have been made to investigate the plant yield increase due to biological fertilizer (inoculation with bacteria), but the role of the PGPB in the different plant development stages remain unknown so far. We hypothesize that the bacterial strains have different mechanisms of plant growth promotion rather than only biological nitrogen fixation, e.g. secretion of phytohormones. Due to the different PGP effects we hypothesize that the colonization behavior and thus the occupation of different ecological niches differs between the bacterial strains. Furthermore, the bacterial activity as well as colonization behavior will be influenced by the plant development stage. Inoculated sugarcane plants are therefore grown under controlled conditions and sampled at different time points of the early plant development stages. To localize the bacteria (fluorescence protein-tagged) in different vegetation states of the plants, samples are analyzed with Confocal Laser Scanning Microscopy to get an insight in the preferential colonization sites. Furthermore, relative abundance quantitative PCR of transcripts of *nifH* and other genes related to PGPR (e.g. expression of genes of phytohormones synthesis) reveals the functional traits of the inoculated bacteria in the plants.

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POSTER 6-17

The Effect of *Azotobacter chroococcum* on Rhizosphere Microorganisms and Sugarbeet Yield in Organic Farming

Nastasija B. Mrkovacki, Dragana Dj. Bjelic, Livija L. Maksimovic

Institute of Field and Vegetable Crops, Novi Sad, Serbia

In systems of sustainable, organic agricultural production, the interaction of plants and microorganisms are very important, primarily in the transformation and mobilization of nutrients from the restricted fund in the soil, then in the plant uptake of these nutrients, in order to realize their full genetic potential (1). Use of microbiological preparations as a supplement or replacement of mineral fertilizers and pesticides become a common practice. The best combination of beneficial bacteria increase the efficiency of these preparations (2). Therefore, the aim of this study was to examine the effect on sugarbeet yield parameters and microbiological soil status using two techniques of sugarbeet inoculation with strains of *Azotobacter chroococcum*.

Cultivar Drena was used in the study, and field trial was set under the conditions of organic farming system in Bački Petrovac. A mixture of three strains of *Azotobacter chroococcum* was used as microbial fertilizer. Inoculation was performed by: incorporation of strains into soil before sowing; and repeated incorporation of strains into soil two weeks after sowing. PGP characterization of the strains confirmed the ability of producing indole-3-acetic acid (IAA) from 12.63 μg ml^{-1} to 14.95 μg ml^{-1}, nitrogen fixation, and P-solubilization. Positive effects on the number of *Azotobacter* sp. and free nitrogen fixers in rhizosphere were obtained by inoculation, as well as positive effects on the tested sugarbeet yield parameters. The largest increase in root yield, yield of crystal sugar, and yield of polarised sugar compared to control was obtained by repeated soil inoculation, ranging from 22 to 23 %.

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POSTER 6-18

The Role of *anf*H and *nif*H on Biological Nitrogen Fixation in the Plant Growth-promoting Bacterium *Kosakonia radicincitans* DSM16656^T

Lempie K. Ekandjo, Rainer Remus, Yvonne Becker, Katja Witzel and Silke Ruppel

Leibniz Institute for Vegetables and Ornamental Crops, Großbeeren, Germany

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Kosakonia radicincitans DSM16656^T is a plant growth promoting bacterium [1]. Its capabilities to colonize both the plant phyllo- and rhizosphere regions and enhance growth of non-leguminous vegetables such as tomatoes (*Solanum lycopersicum*), radish (*Raphanus sativus*), and *Brassica oleracea* in greenhouses as well as in fields [2, 3] upon inoculations, maximize its potential as a bio-inoculant in modern agricultural systems. Biological nitrogen fixation (BNF) might be essential in yield increases in non-leguminous inoculated plants. *K. radicincitans* has the MoFe-nitrogenase encoded by the *nifHDK* genes and the FeFe-nitrogenase encoded by the *anf*HDGK genes. To investigate the role of these two nitrogenase systems of this strain in the observed growth-promoting effects in plants, site directed mutation was conducted to delete *nif*H and *anf*H and create single and double mutants of *K. radicincitans* DSM16656^T that were used in a $^{15}N_2$ isotopic labelling experiments. *K. radicincitans* wild-type; Δ*nif*H; Δ*anf*H; and Δ*nif*HΔ*anf*H mutants were cultured in semi-solid molybdenum containing nitrogen-free medium in conical flask. Half of the flasks were incubated with room air and the other half with 6.5 L labeled $^{15}N_2$ plus 1.73 L O_2 for 7 days at room temperature, prior to sample collection. For analysis, lyophilized samples were combusted in an elemental analyzer and ^{15}N abundance was measured in a NOI-6 PC emission spectrometer. Our data demonstrate that under the applied conditions only *nif*H, but not *anf*H does have an impact on BNF in *Kosakonia radicincitans* DSM16656^T. Our results suggest that the FeMo-nitrogenase is the key player nitrogenase in *K. radicincitans* DSM16656^T.

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POSTER 6-19

Transcriptomics for Deciphering Actinorhizal Symbiosis

Philippe François[1,2], Cyrille Longin[2], David Vallenet[2], Stéphane Cruveiller[2], Sergio Svistoonoff[1,3], Hassen Gherbi[1], Didier Bogusz[4], Valérie Hocher[1]

[1] *IRD, UMR LSTM, TA A-82/J, Campus International de Baillarguet, Montpellier, France*
[2] *CEA/DSV/IG/Genoscope-CNRS UMR8030, Laboratoire de Génomique Comparative (LGC), Evry, France*
[3] *Laboratoire Commun de Microbiologie IRD/ISRA/UCAD, Centre de Recherche de Bel Air, Dakar, Senegal*
[4] *IRD, UMR DIADE, Centre IRD de Montpellier, Montpellier, France*

Nitrogen-fixing root nodulation, confined to four plant orders, encompasses more than 14,000 Leguminosae species, and approximately 200 actinorhizal species forming symbioses with *rhizobia* and *Frankia* bacterial species, respectively. Legumes and actinorhizal plants all belong to the Fabid clade suggesting a common ancestor having acquired a genetic predisposition towards nodulation (1). Some molecular actors of this predisposition could have been recruited from the more ancient Arbuscular Mycorrizae (AM) endosymbiosis (2). Recent years have witnessed intense growth of different genomic approaches (ESTs, microarray, and deep sequencing) for high-throughput analyses of genes and their transcripts. This was recently applied to different actinorhizal systems. Most of the genes of the common "SYM" pathway described for AM and legumes-rhizobium symbioses (3, 4) were identified in actinorhizal symbiosis Interestingly, our analysis also revealed the presence of genes linked to a "NOD"-specific pathway (not shared with AM symbiosis) as used by legumes in their symbiosis with rhizobia (5).

To target genes involved during early events of symbiosis (pre-infection, infection), we developed a high throughput-sequencing program (RNA-Seq) on *Casuarina*. A *de novo* transcriptome was sequenced and gene expression analysis (RNA Seq) will be now conducted in *Casuarina* roots during a kinetic of inoculation by *Frankia*. Results of this study will be presented and their impact for understanding root endosymbioses evolution will be discussed.

A WEB platform integrating all these data will be presented and will be made available to scientific community in order to distribute knowledge related to actinorhizal symbioses worldwide. Last, future development on actinorhizal plants genomic will be presented.

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POSTER 6-20

Use of Trap Plants to Isolate PGPB Strains from Soils under Different Land Uses and Its Application as Inoculant for Non-legumes

André L. Martinez Oliveira, Mónica Y. Alzate-Zuluaga, Karina M. Lima Milani, Odair José A. P. Santos, Amanda Aleixo, Danielle Cristina Ferreira, Karita dos Reis Costa

Departamento de Bioquímica e Biotecnologia, Universidade Estadual de Londrina - UEL, Londrina, Brazil

The soil microbiome is strongly modulated by the quality and quantity of root exudates, a phenomenon known as 'rhizosphere effect' that induces increase in the population density of few bacterial taxa, while lowers the bacterial diversity found in the non-rhizospheric soil (1). This phenomenon was explored by growing maize or tomato in soils from different land uses following the isolation of nitrogen-fixing bacteria able to colonize these plants in population densities in excess of 1 x 10^5 cells g^{-1} root+rhizosphere. Representatives of the culturable pool from the root+rhizosphere residing bacteria were characterized (biochemical traits) and phylogenetically positioned (16S rRNA gene sequencing) to identify potential plant growth-promoting bacteria (PGPB). Inoculation trials under greenhouse and field conditions were performed to confirm the growth-promoting ability of selected strains. The bacterial community accessed from maize plants comprised 107 diazotrophic isolates, mainly from the Proteobacteria domain (87% of isolates). From these collection, 54 isolates promoted the initial development of maize plants (10 days of growth, seed-inoculated plants) while 8 isolates decreased the plant development. 17 isolates improved the initial growth of maize plants in up to 30% (compared to uninoculated plants), following the evaluation under greenhouse conditions. These strains were used to prepare liquid and solid inoculant formulations, this last using as vehicle a biodegradable foam prepared by extrusion of a starch/cane bagasse-based mixture. From the isolates tested under field conditions so far, three strains showed consistent results as PGPB for maize: *Rhizobium* sp. strain 8121, *Pseudomonas* sp. strain 4331, and *Azomonas* sp. strain 4311. Among other effects observed in the physiology and development of plants, maize inoculation with the selected strains could substitute up to 70% of maize N-fertilizer demand without decrease the productivity. In this same sense, the use of tomato as trap-plant to access the soil bacterial community with plant growth-promoting potential resulted in 49 bacteria isolates comprising 8 different genera: *Rhizobium* (32 isolates), *Variovorax* (5 isolates), *Burkholderia* (3 isolates), *Pseudomonas* (3 isolates), *Caulobacter* (1 isolate), *Herbaspirillum* (1 isolate), *Massilia* (1 isolate) and *Roseateles* (1 isolate). Although the use of tomato as trap-plant resulted in a lower diversity of isolates as compared to maize, we found 16 bacterial strains that increased the dry weight of tomato seedlings in up to 30%, from which four bacterial strains (*Pseudomonas* sp. strain 17T, and *Rhizobium* sp. strains 4T, 21T and 41T) were selected and are under evaluation in a greenhouse trial to confirm its growth-promoting ability. In conclusion, the approach adopted to access soil bacteria by the use of trap-plants expanded the knowledge of the PGPB diversity in Brazilian soils, indicating new potential strains to produce commercial inoculants for maize and highlighting the putative role of Rhizobium as associative bacteria in non-leguminous plants as tomato.

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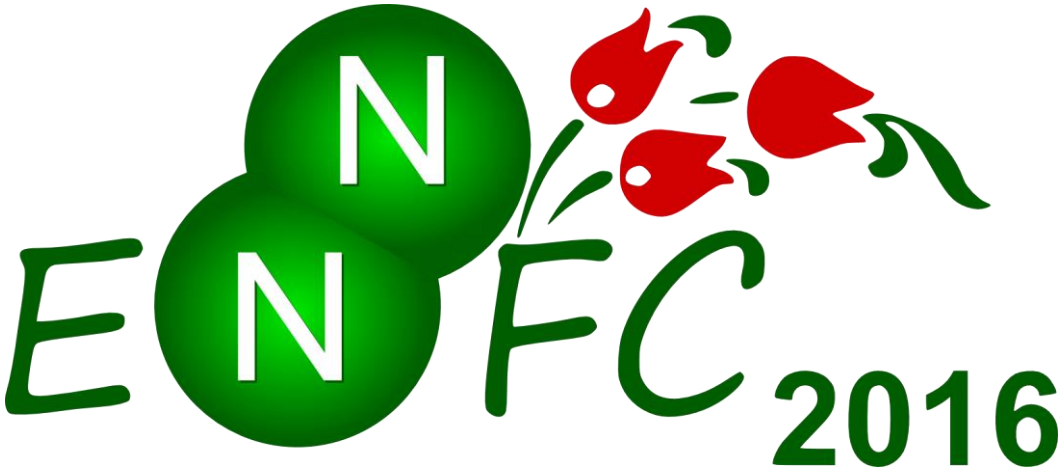

POSTER SESSION 7

Free-living Nitrogen Fixation

Room Helia

POSTER 7-1 /LIGHTNING TALK/

Isolation and Characterization of Two New Nitrogen Fixing Unicellular Cyanobacteria from the Indian Ocean

Sofie Vonlanthen and Rachel A. Foster

Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm, Sweden

In the marine environment, cyanobacteria are often considered the most important nitrogen fixing organisms, providing new bioavailable nitrogen to the oligotrophic oceans. Although larger filamentous and heterocyst forming symbiotic species, such as ***Trichodesmium*** and ***Richelia*** have previously been considered the dominant nitrogen fixers in the open ocean, more recent studies have found that unicellular cyanobacteria play an equally important role (1). The unicellular cyanobacteria group B (UCYN B), represented by the cultured strain ***Crocosphaera watsonii***, is widely distributed across tropical and subtropical oligotrophic oceans (2). Six strains have been isolated from the Pacific and the Atlantic Oceans and whole genome comparisons have shown surprisingly low genetic variation between strains, despite phenotypic variations (3). Other known unicellular cyanobacteria include group C, closely related to the costal and benthic species of Cyanothece (4). Isolated strains of Crocosphaera are limited to two oceans (Atlantic and Pacific) and to further investigate the diversity of unicellular nitrogen fixing cyanobacteria we aimed to isolate new strains, from the previously under sampled Indian Ocean.

Two new strains (SU2 and SU3) of unicellular cyanobacteria were isolated from the waters outside Zanzibar, Tanzania. Both strains have a coccoid or oval shape measuring 3-5µm and grow in medium lacking combined nitrogen.

Phylogenetic analysis of the 16srRNA and nifH genes show that one strain (SU2) is more closely related to C. watsonii (96% and 91%, respectively) and one (SU3) is more closely related to Cyanothece (99% and 98% respectively).

Both strains produce large amounts of extra cellular polysaccharides as imaged by Alcain blue staining and microscopy, and SU3 form large visible colonies comprised of hundreds of cells. Experiments to measure Carbon and Nitrogen fixation were performed with $^{15}N_2$ and ^{13}C-bicarbonate. Both bulk and single cell rates will be presented from analysis using secondary ion mass spectrometry (SIMS). In addition, whole genome sequencing was performed on both species using Illumina Miseq. The GC content of SU2 and SU3 is 40.1 and 36.6% respectively. SU2 and SU3 represent the first two isolated strains of diazotrophic unicellular cyanobacteria from Indian Ocean.

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POSTER 7-2 /LIGHTNING TALK/

Molybdenum Metabolism in *Azotobacter vinelandii*

Mónica Navarro-Rodriguez, Emilio Jiménez-Vicente and Luis M. Rubio

Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid, Spain

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Azotobacter vinelandii is a model organism for biochemical studies of N_2 fixation that has the peculiarity of being able to fix N_2 under aerobic conditions. This process could be carried out via three homologous nitrogenases that differ in the heterometal found at their active sites: the Mo nitrogenase, the V nitrogenase and the Fe-only nitrogenase (1). Expression of either Mo nitrogenase or the alternative nitrogenases depends on the metal availability in the medium, with molybdate acting as co-repressor of alternative nitrogenases. In addition, *A. vinelandii* carries a molybdenum storage protein, referred to as MoSto, which can store up to 25-fold more Mo than needed during maximum nitrogenase activity (2,3). In this work we investigate a plausible role of MoSto as an obligate intermediate in the Mo trafficking pathway that provides molybdenum for the biosynthesis of the iron-molybdenum cofactor (FeMo-co) of the Mo nitrogenase. We analyze the phenotype of an *A. vinelandii mosAB* in-frame deletion mutant strain, which lacks the genes encoding the MoSto subunits. Wild type and mutant strains are compared in: (*i*) their ability to grow diazotrophically and non-diazotrophically in media containing decreasing amounts of molybdate; (*ii*) their levels of intracellular Mo; (*iii*) the *in vivo* levels of Mo nitrogenase activity; (*iv*) their capacity to repress expression of the alternative nitrogenases.

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POSTER 7-3 /LIGHTNING TALK/

Studies of DraB, a Small Thioredoxin Like Protein in *Rhodospirillum rubrum* with an Unknown Function Encoded Within the *dra* Operon

S. Gudise, A. Bock, S. Rosowski, S. Nordlund, Agneta Norén

Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden

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Nitrogen fixation is studied at the molecular level in the free-living diazotroph, *Rhodospirillum rubrum,* where nitrogenase activity is controlled at a post- translational level with signalling pathways transducing changes in nitrogen/energy status. The pathway for energy (and nitrogen) signalling includes 2 regulatory proteins DraT and DraG in our model organism. These proteins are involved in reversible ADP ribosylation of nitrogenase that regulates nitrogenase activity. We have earlier shown that this involves protein-protein interactions and changes in cellular localization of DraG as a response to high ammonia conditions. Presently the energy signalling pathway is our focus and we have shown that changes in energy supply do lead to association of DraG to the membrane in *R. rubrum*, both when the light is turned off in photoheterotrophically grown cells and when oxygen is removed in nitrogen-fixing cultures grown aerobically in the dark. In *R. rubrum* DraT and DraG are encoded in an operon where an additional gene, *draB,* encoding a protein with unknown function is present. The expression, purification and localization of DraB and construction of a *draB* mutant is in progress. The primary sequence of DraB reveals high similarity to proteins in the thioredoxin family thereby indicating a possible role in redox reactions. DraB is now to be characterized and further investigated for a possible role the regulation inresponse to cellular energy status. The redox changes in our model organism can be manipulated by light/darkness, oxygen concentration and growth on different carbon sources.

The methods used include basic DNA overexpression systems with tagged target proteins (FLAG, his, GST) for further purification and detection. Protein protein interactions will be investigated by immunoprecipitation. Localization studies include cell fractionation along with Western blot analysis. A *draB* mutant is constructed by the insertion of an antibiotic cassette in the gene and further transformed into an *Eschrichia coli* S-17 strain for conjugation with *R.rubrum* to create a *draB* mutant. In addition for elucidate DraB properties spectroscopy, crystallization and various biochemical methods are used.

POSTER 7-4

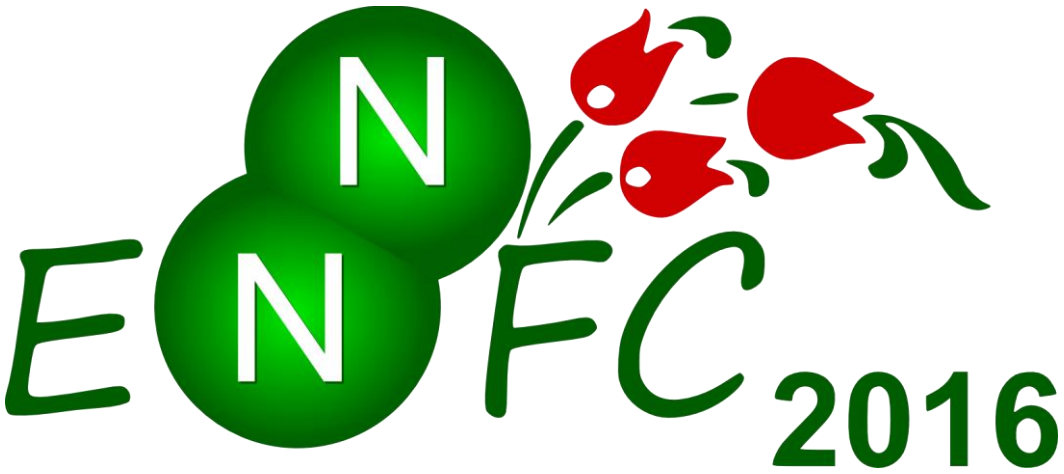
Exploring Diazotrophic Growth Processes in Marine Cyanobacteria using Combined, Experimental and Modeling Approaches

Sophie Rabouille

CNRS- UMR7093, Laboratoire d'Oceanographie de Villefranche, Villefranche-sur-Mer, France
srabouille@obs-vlfr.fr

The discovery of new diazotrophic organisms in the world's ocean has widened the puzzle of the oceanic, nitrogen budget. Experiments performed on the planktonic, cyanobacterial strains cultured to date have shed some light on their respective physiological properties and growth efficiencies. Even though these strains are only a few amongst the wide diversity of nitrogen fixers, they do take a share in the global marine primary production. But how significant is this share in the world ocean? Global scale modeling approaches are best suited to tackle this question; yet current biogeochemical models fail in predicting observed rates. This inaccuracy is likely to be related to the very simplistic representation of nitrogen fixation. With the aim to analyze and discuss what level of information matters that should be incorporated in biogeochemical models, we developed models of nitrogen fixation at the organism scale to describe growth processes in response to environmental conditions. These models are used as virtual laboratory and compared to culture data to gain better understanding of the metabolic controls of nitrogen fixation.

Keywords: nitrogen fixation, physico-chemical forcing, cultures, modeling.



POSTER SESSION 8

On the Interface of Symbiotic/Pathogenic Interactions

Room Uranus

POSTER 8-1 /LIGHTNING TALK/

Symbiosis or Defense: The Molecular Mechanism Involving LysM Receptors of the Model Legume *Lotus japonicus*

Zoltán Bozsóki[1], Jeryl Cheng[1], Mickael Blaise[2], Jens Stougaard[1], Simona Radutoiu[1]

[1] *Centre for Carbohydrate Recognition and Signalling (CARB), Department of Molecular Biology and Genetics, Aarhus University, Aarhus, Denmark*
[2] *Centre d'études d'agents Pathogènes et Biotechnologies pour la Santé, CNRS, Université de Montpellier, Montpellier, France*
zoltanbo@mbg.au.dk

Chitin oligomers derived from fungal cell walls are recognized as PAMPSs and elicit defense responses in plants. On the other hand symbiotic nitrogen fixing rhizobia bacteria produce signaling molecules with a chitin backbone that are essential for the symbiosis initiation (Nod factors). LysM motif containing receptors have been shown previously to be essential for perception of chitin-backbone-containing molecules. Rice CEBiP and Arabidopsis CERK1 are indispensable for chitin elicited defense signaling, while legumes mutated in *L. japonicus* NFR1 and NFR5 are unable to respond to Nod factors and symbiotic bacteria. We have identified and characterized the chitin receptor in *L. japonicus*, LYS6. *lys6* mutant plants fail to respond to chitin oligomers with oxidative burst, MAPK3/6 phosphorylation or defense gene activation, but are able to form efficient symbiosis with rhizobia and arbuscular mycorrhiza. On the other hand, *nfr1* mutants respond normally to chitin oligomers. NFR1 and LYS6 share 78% similarity in their amino acid sequences, are expressed in the same type of root cells, yet drive signaling pathways leading to opposing outcomes. We aim to identify molecular patterns present in the two receptors responsible for the symbiosis/defense decision at the very first steps of microbe recognition to broaden our understanding on the function and evolution of LysM receptor-mediated signaling in root cells. For this we use single and double mutant phenotypic analyses coupled with genetic complementation studies using synthetic receptor molecules. The results of our analyses will be presented and discussed.

POSTER 8-2 /LIGHTNING TALK/

Assessing the Relevance of a Range of Polysaccharide Signaling Molecules for Activation of Symbiotic Signaling

Jongho Sun[1], Feng Feng[1], Sarah Shailes[1], Simona Radutoiu[2], Jens Stougaard[2] and Giles Oldroyd[1]

[1] *John Innes Centre, Norwich, United Kingdom*
[2] *Department of Molecular Biology and Genetics, Centre for Carbohydrate Recognition and Signalling, Aarhus University, Denmark*

Plants associate with micro-organisms to facilitate nutrient acquisition, with most plants interacting with AM fungi, but only a few plants, such as legumes, associating with nitrogen-fixing rhizobial bacteria. Both AM fungi and rhizobia signal to the plant via diffusible lipochitooligosaccharides (LCOs) or chitooligosaccharides (COs) signaling molecules that can activate the common symbiosis signaling pathway with induction of calcium oscillations. Activation of symbiosis signaling by LCOs is mostly restricted to legumes, while perception of COs during symbiosis appears to occur more broadly within the plant kingdom. Recently, we have found that long-chain chitin oligomers CO8 can trigger not only ROS production and MAPK phosphorylation but also symbiotic calcium oscillations. In contrast, CO4 activates symbiotic calcium oscillations, but not PAMP responses. Both CO4 and CO8 activate symbiotic calcium oscillations in a range of plant species. These results indicate that CO8 is involved in both symbiotic and immune signaling, while CO4 appears to be limited to symbiotic signaling. Reverse genetic screening in both *Lotus japonicus* and *Medicago truncatula* revealed multiple LysM-RLK mutants with defects in CO8-induced PAMP and CO8/CO4 induced symbiosis signaling. These chitin receptors are closely related to, but different to the Nod factor receptors associated with perception of rhizobia. Our results suggest a complex of LysM-RLKs in legume roots that function in chitin perception and a separate complex of LysM-RLKs associated with Nod factor perception. While both complexes appear to function in symbiosis signaling, only the CO receptor complex also activates PAMP signaling.

POSTER 8-3 /LIGHTNING TALK/

The Investigation of the Mechanisms by which Pea Plants Discriminate and Respond to Structurally Related COs Signals from Symbiotic and Pathogenic Fungi

Irina Leppyanen, Nadezhda Vishnevskaya, Oksana Shtark, Vlada Shakhnazarova, Elena Dolgikh

All-Russia Research Institute for Agricultural Microbiology (ARRIAM), Saint Petersburg, Pushkin, Russia

Plants are able to effectively detect long-chain chitooligosaccharides (COs) released from the cell walls of pathogenic fungi that leads to defence reactions and inactivation of the potential pathogen (1-2). Structurally related short-chain COs (n = 4, 5), produced by mycorrhizal fungi, elicit symbiotic responses in plant roots leading to the symbiosis development (3). How plants perceive the structurally related CO signaling molecules to elicit defence and symbiotic responses respectively is the major objective of our work. Recently the LysM-containing receptor-like protein CERK1 was shown to have dual functions in symbiosis and immunity signaling and involved in COs perception in rice (4). To find out how pea plants *Pisum sativum* L. are able to distinguish COs signaling molecules with similar structure, the searching of CERK1-like receptor has been performed. As a result, the pea LysM-receptor kinase LYK9 was shown to be required for response to treatment with COs having various degree of polymerization. Treatment of pea plants with COs (n = 8-10) resulted in increased level of *PsLyk9* gene expression together with activation of defense genes markers (*PsPAL1*, *PsPAL2* and *PsPR10*). In addition, plants with suppression of *PsLyk9* gene expression using RNA interference were more susceptible to infection with low pathogenic fungus *Fusarium culmorum* (Wm.G.Sm.) Sacc. 891. Reduced disease resistance correlated with decreasing of genes expression encoding defense proteins and enzymes (*PsPR10*, *PsPAL2*). Moreover, in plants with repressed of *PsLyk9* expression treated by short-chain COs (n=5) a significant decrease in the expression of marker genes of arbuscular mycorrhizal symbiosis (*DELLA*, *NSP2*, *DRP* and *RAM1*) has been observed. These results allow us to conclude that the *Ps*LYK9 is the most likely CERK1 receptor homolog in pea that is involved in the control of plant immunity and AM symbiosis formation making the complexes with various co-receptors.

This work was supported by the Russian Scientific Foundation (RSF project no. 16-16-10043).

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POSTER 8-4 /LIGHTNING TALK/

Hopanoids Play an Important Role in *Bradyrhizobium* Strains during their Free-living and Symbiotic States

Nicolas Busset[1], A. Silipo[2], G. Kulkarni[3], D. Gully[1], C. Chaintreuil[1], J. Fardoux[1], D. Gargani[4], F. Gressent[1], A. Molinaro[2], D. K. Newman[3], E. Giraud[1]

[1] *IRD, UMR LSTM, Montpellier, France*
[2] *Dipartimento di ScienzeChimiche, Università di Napoli Federico II, Naples, Italy*
[3] *Division of Biology and Biological Engineering, CIT, Pasadena, United States*
[4] *CIRAD, UMR BGPI, Montpellier, France*

Unlike other rhizobia, the outer membrane (OM) of *Bradyrhizobium* strains contains pentacyclic triterpenoids called hopanoids, which can represent up to 40% of total lipids (1). These molecules, which display structural similarity with eukaryotic sterols, constitute an important class of membrane lipids widely distributed in diverse bacteria. Hopanoids are thought to act as membrane condensers, thus increasing bacterial resistance to various abiotic stresses. Interestingly, two classes of hopanoids are present in the OM of *Bradyrhizobium* strains, classical "free" hopanoids such as diploptene, diplopterol or tetrahymanol and a novel class corresponding to a C_{35} hopanoid molecule called bacteriohopanetetrol which is covalently linked to the lipid A and named HoLA (2). Here we investigate the role of hopanoids by mutating some genes involved in hopanoids biosynthesis in two *Bradyrhizobium* strains, the photosynthetic strain BTAi1 and the non-photosynthetic strain *B. diazoefficiens*.

In the strain BTAi1, a complete hopanoid deficient mutant was built by mutating the squalene hopene cyclase gene (*shc*) which is a key enzyme involved in the cyclisation of the squalene into hopanoid. Analysis shows that the mutant is more sensitive to various stresses under free-living conditions. On *Aeschynomene* plants, the *shc* mutant induces functional nodules but with a life span drastically reduced indicating an important role of hopanoids for maintaining chronic intracellular infection (2).

In the strain *B. diazoefficiens,* we were unable to obtain an *shc* mutant. This suggests that hopanoids synthesis is essential for growth and survival of *B. diazoefficiens*. However, we succeeded to obtain two other mutants in the two most important hopanoids family, 2-methylated (2Me) and extended (C_{35}) hopanoids by deleting respectively the *hpnP* and *hpnH* genes (3). Our results indicate that both classes of hopanoids play an important role in free-living state under various stress conditions. When the mutants were tested on plant, no effect of the *hpnP* mutation was observed, while the *hpnH* mutant was found to be altered for several aspects of the symbiosis, including evasion of plant defense reactions, or nitrogen fixation in symbiosis with *A. afraspera* but not with soybean. The difference observed between the two hosts for the *hpnH* mutant is likely related to the presence of cysteine-rich antimicrobial peptides in *Aeschynomene* nodules that induce drastic modification in bacterial physiology (3).

In conclusion, hopanoids play an important role to optimize bacterial survival in both free-living and symbiotic states of *Bradyrhizobium* strains.

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POSTER 8-5 /LIGHTNING TALK/

Unraveling Plant Cellular Targets for the *Rhizobium*-specific Effectors NopL and NopP

I. Jiménez-Guerrero[1], F. Pérez-Montaño[1], C. Medina[2], E. Santero[2], J. A. Monreal[3], F. J. Ollero[1] and Francisco J. López-Baena[1]

[1] *Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla. Sevilla, Spain*
[2] *Departamento de Biología Molecular e Ingeniería Bioquímica, CABD, Universidad Pablo de Olavide/CSIC/Junta de Andalucía, Sevilla, Spain*
[3] *Departamento de Fisiología Vegetal, Facultad de Biología, Universidad de Sevilla. Sevilla, Spain*

Pathogenic Gram-negative use a specialized apparatus called Type 3 secretion system (T3SS) to deliver effectors directly into the eukaryotic host cells. These effectors suppress plant defenses to promote disease but they can also be recognized by specific plant receptors that trigger a strong defense reaction to eliminate the pathogen (1). The T3SS has also been found in some symbiotic rhizobial strains and the effectors secreted are involved in host-range determination and symbiotic efficiency.

The broad host-range bacterium *S. fredii* HH103 secretes two proteins through the T3SS, NopL and NopP, which are specific for rhizobia. In this work we studied the function of both effectors in the symbiosis with soybean, which is considered its natural host plant. NopL and NopP were phosphorylated by soybean root kinases and the phosphorylation cascade was Ca^{2+}- and calmodulin-dependent. While the signaling pathway that culminates in the phosphorylation of NopL included ser/thr and MAPKK kinases, in the case of NopP this pathway involved ser/thr and tyr kinases but not MAPKK kinases.

Transient expression of both *nopL* and *nopP* fused to YFP in *Nicotiana benthamiana* leaves and further confocal imaging indicated that they localized to the nucleus of the host cell. The use of a yeast-based array to determine possible effectors functions indicated that NopP could be involved in nuclear localization and migration. Finally, co-immunoprecipitation analyses of *N. benthamiana* NopL- and NopP-interacting proteins and further mass spectrometry analyses identified several potential plant targets for these effectors. The most interesting interactions are currently being validated by Bimolecfular Fluorescence Complementation (BiFC)

This work was supported by project P11-CVI-7050 of the Junta de Andalucía.

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POSTER 8-6 /LIGHTNING TALK/

Specialised Protein Secretion in Plant-Microbe Symbioses

Onur Oztas[1], Huairong Pan[1], Minsoo Kim[1], Christina Stonoha[1], Xiaoyi Wu[2], Bin Wang[2] and Dong Wang[1]

[1] *University of Massachusetts, Amherst, MA, United States*
[2] *Nanjing University, Nanjing, China*

Legumes can engage rhizobia, transforming free-living bacteria into nitrogen-fixing organelles (symbiosomes). This symbiosis co-opts the machinery of a more widespread association with arbuscular mycorrhizal (AM) fungi[1]. Both symbionts are outlined by a specialized interfacial membrane, which is derived from the host plasma membrane but maintains its unique identity. The protein secretory pathway has been co-opted to deliver host factors to the microsymbiont[2,3]. We recently discovered that the fidelity of this symbiosis-specific protein secretion is insured by a t-SNARE protein generated by a transcriptional regulatory mechanism called alternative cleavage and polyadenylation, and that this symbiotic t-SNARE is crucial to a properly developed symbiosis[4].

Unlike AM fungi, rhizobia exist intracellularly through a persistent infection. Sustaining and controlling such a chronic infection is a major challenge to the host, and could be a key factor in the emergence of the nitrogen-fixing symbiosis. We discovered that to insure the survival of newly internalized rhizobia, the host processes certain symbiosome membrane proteins to suppress excessive defence responses. As bacteroids mature, a new set of factors is needed to maintain the viability of such enlarged bacteria. We discovered that one of the Nodule-specific Cysteine-Rich (NCR) peptides is required for the survival of differentiated bacteroids[5]. Current evidence suggests this NCR peptide localizes to the bacterial surface, and could promote bacteroid survival by facilitating metabolite exchange between the host and the bacteria.

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POSTER 8-7 /LIGHTNING TALK/

Rhizobia Inoculation Reduces *Didymella pinodes* Impacts on Photosynthetic Efficiency of *Pisum sativum*

Getinet Desalegn[1], R. Turetschek[2], H.-P. Kaul[1], S. Wienkoop[2]

[1] *Department of Crop Sciences, University of Natural Resources and Life Sciences, Tulln, Austria*
[2] *Department of Ecogenomics and Systems Biology, University of Vienna, Vienna, Austria*

The needs for sustainable crop production systems and plant-based protein for both human and animal consumption are rising globally. In these aspects, field pea (*Pisum sativum* L.) often plays an important role due to its ability to fix atmospheric nitrogen and high protein content. It can also form symbiotic association with **arbuscular mycorrhiza (AM) fungi which facilitate plant uptake and transport of relatively immobile soil nutrients such as phosphorus and others.** However, the global expansion of *P. sativum* production has major abiotic and/or biotic impediments, of which *Didymella pinodes* is a very damaging foliar disease (1, 2) through reduction of photosynthetic efficiency of pea plants. Elsewhere up to 70% yield losses of peas caused by this pathogen have been reported. On the other hand, recent evidences showed that a type of root-associated microbes that increase plant growth and yield, elicit induced systemic resistance against pathogens (3, 4, 5). We evaluated the effects of below ground rhizobia and AM fungi **inoculations on photosynthetic efficiency and production of dry matter of two field pea genotypes (cultivar Messire and Protecta). We found that** there were significant differences between some of the group averages of pea plants **in green area, leaf greenness, photosynthetic efficiency and shoot dry matter production. T**he highest pathogen infection and lowest photosynthetic components such as leaf greenness and green areas were observed in susceptible cultivar Messire in which about **50% green area reduction was found as compared to healthy plants. Under pathogen attack, the rhizobia increased more dry matter production than AM fungi by 23%.** Furthermore, the lowest pathogen infections and highest photosynthetic efficiency results were recorded with cultivar Protecta inoculated with *Rhizobium*.

Overall, we conclude that the belowground rhizobial and AM fungi symbiotic associations enhance significantly photosynthetic efficiencies and production of shoot biomass compared to the control treatment in both *P. sativum* cultivars under *D. pinodes* attacks.

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POSTER 8-8

A Key Plant Immune Protein is Essential for the Legume-rhizobium Symbiosis

Katalin Tóth and Gary Stacey

Divisions of Plant Sciences and Biochemistry, C. S. Bond Life Sciences Center, University of Missouri-Columbia, Columbia, United States

A paradigm shift is occurring in the field of symbiotic nitrogen fixation with a growing realization of the central importance of the plant immune response in the earliest steps of rhizobial infection and establishment of the symbiosis. Our data describe novel and important findings that, for the first time, provide a mechanistic link between legume-rhizobia symbiosis and plant immunity.

In order to better understand the biology of this mutualistic interaction, we conducted a phosphoproteomic study on soybean root and root hairs in response to the compatible symbiont *Bradyrhizobium japonicum*. A protein well characterized in plant immune responses was found to be phosphorylated within one hour post-inoculation in soybean root hairs in response to *B. japonicum*. Silencing of this gene using RNAi resulted in a significant reduction in nodule formation. One of the identified phosphorylation sites was previously described as responsive to MAMP (Microbe-Associated Molecular Pattern) treatment in Arabidopsis. We introduced a phosphomimetic (a mutation to D) and phospho-minus (a mutation to A) point mutations in the gene and constitutively expressed the mutated protein in soybean transgenic roots. The expression of the phosphomimic version resulted in a significant reduction in nodule formation, while expression of the dephosphorylated version did not significantly impact nodulation. On this same protein, a second phosphorylation site was identified, located within a 15 amino acid region, which appears to be present only in proteins derived from leguminous plants. When a phospho-minus version of this phosphorylation site was introduced into transgenic soybean roots, significantly fewer nodules were formed, suggesting that the site might be required for the symbiotic signaling. Our preliminary data suggest that phosphorylation of at least one of the identified sites is triggered in response to treatment by the rhizobial signaling molecule, Nod factor. The data provide evidence for a key regulatory link between the Nod factor signaling pathway and well characterized pathways involved in plant innate immunity.

POSTER 8-9

Analysis of a *Medicago truncatula* Mutant Showing Induced Defense Responses in Symbiotic Nodules

Mónika T. Tóth, Gy. Kováts, A. Gombár, L. Fodor, B. Horváth, P. Kaló, Á. Domonkos

Agricultural Biotechnology Institute, NARIC, Gödöllő, Hungary

Ineffective symbiotic nitrogen fixing (Fix-) *Medicago truncatula* mutants were characterized to identify plant genes required for rhizobial invasion and differentiation to bacteroids. We have isolated an ineffective symbiotic mutant which developed yellowish-white nodules and displayed symptoms of nitrogen starvation. The cross-sections of mutant nodules showed strong pigmentation 6-8 days after infection with rhizobia and corresponding to this region, the autofluorescence of the mutant nodules was detected indicating the induction of defense response in the non-functional nodule. Based on the detected nodule phenotype, we termed the mutant *induced defense response* (*idr*). Further phenotypic analysis revealed that the appearance of characteristic pigment accumulation was irrespective of the rhizobial strains (*S. meliloti* 1021, 2011 and ABS7, *S. medicae* WSM419) used for inoculation. Monitoring of the expression of pathogen-related genes revealed their increased transcript level confirming the induction of defense responses. The analysis of the expression pattern of the symbiosis specific genes and the presence of the rhizobia in the mutant nodules indicated that *IDR* probably functions following the bacterial invasion of the nodule and preceding the onset of bacteroid differentiation. The analysis of the activity of the *IDR* promoter using the GUS reporter gene demonstrated that *IDR* is expressed in the interzone and nitrogen fixation zone of the symbiotic nodules. A map-based cloning project identified the IDR gene that encodes a small peptide with unknown function. The analysis of the sequence of the *IDR* gene detected a 50bp deletion in the *idr* mutant plant and the introduction of the wide type *IDR* gene into the mutant proved the gene identity. In order to analyze the position of the *IDR* gene in the hierarchy of the symbiotic genes, double mutants of *IDR* and other late symbiotic genes (*LIN*, *DNF1*, *DNF2*, *DNF5*) were generated and analyzed for their symbiotic phenotype following rhizobium inoculation.

POSTER 8-10

Comparative Analysis of the Bacterial Membrane Disruption Mechanism of Two Natural *Medicago truncatula* Antimicrobial Peptides

Attila Farkas, Attila Kereszt, Éva Kondorosi, Gergely Maróti

Institute of Biochemistry, Biological Research Centre HAS, Szeged, Hungary

Legumes are unique in their ability to establish symbiotic interactions with *Rhizobium* bacteria and develop a new organ, the nitrogen-fixing root nodules. In the nodule, inside the plant cells bacteria irreversibly convert to polyploid, non-dividing nitrogen-fixing bacteroids that can reduce atmospheric nitrogen to ammonia. In IRLC legumes terminally differentiated bacteroids are controlled by host symbiosis specific plant peptides (1). Approximately 800 genes code for nodule-specific cysteine-rich (NCR) peptides in the *Medicago truncatula* genome. The NCR peptides are composed of a conserved signal peptide and a highly variable 20-50 amino acid long secreted peptide with four or six conserved cysteine residues (2).

NCR247 and NCR335, representatives of the most cationic members of the family exhibit *in vitro* antimicrobial activity against Gram+ and Gram- bacteria including important pathogens (3). Investigation of the symbiotic function of NCR247 revealed that the peptide can enter the endosymbionts without membrane damage and interact with multiple bacterial targets (4). On the other hand killing action of cationic antimicrobial peptides (AMPs) is most frequently achieved by disruption of bacterial membranes. As membrane disruption can be strongly dependent on peptide concentrations, the bactericidal effect of NCR247 and NCR335 was studied on *Salmonella enteritidis* and *Listeria monocytogenes* as Gram+ and Gram- model bacteria and were compared to that of Polymyxin B (PMB), a cyclic AMP and streptomycin (STR). *S. enteritidis* was sensitive to NCR247, NCR335 and PMB and resistant to STR while *L. monocytogenes* was only sensitive to NCR335. NCR247 and NCR335 accumulated in the cytosol of *S. enteritidis* while in *L. monocytogenes* they were mostly observed in the bacterial membrane. Scanning electron microscopy of peptide-treated bacteria revealed different membrane effects. Leakage and complete cell disruption of *S. enteritidis* by PMB and NCR335 and budding of the cell surface by NCR247 were detected. PMB had no effect on *L. monocytogenes* while the cell content was released by NCR335 and NCR247 provoked only mild morphological changes. This comparative analysis reveals that membrane effects of AMPs in addition to their charge depend also on their primary sequences and on the target bacteria.

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POSTER 8-11

Effector-triggered Immunity Determines Host Genotype-specific Incompatibility in Legume-*Rhizobium* Symbiosis

Michiko Yasuda[1], Hiroki Miwa[1], Sachiko Masuda[1], Yumiko Takebayashi[2], Hitoshi Sakakibara[2], Shin Okazaki[1]

[1] *International Environmental and Agricultural Science, Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan*
[2] *Plant Productivity Systems Research Group, RIKEN Center for Sustainable Resource Science, Yokohama, Japan*

Symbiosis between legumes and rhizobia leads to the formation of N_2-fixing root nodules. In soybean, several host genes, referred to as *Rj* genes, control nodulation. Soybean cultivars carrying *Rj4* gene restrict nodulation by specific rhizobia such as *Bradyrhizobium elkanii*. We previously reported that the restriction of nodulation was caused by the *B. elkanii* possessing a functional type III secretion system (T3SS), which is known for its delivery of virulence factors by pathogenic bacteria. In the present study, we investigated the molecular basis for the T3SS-dependent nodulation restriction in *Rj4*-soybean. Inoculation tests revealed that soybean cultivar BARC-2 (*Rj4*/*Rj4*) restricted nodulation by *B. elkanii* USDA61, whereas its nearly isogenic line BARC-3 (*rj4*/*rj4*) formed nitrogen-fixing nodules with the same strain. Root-hair curling and infection thread were not observed in the roots of BARC-2 inoculated with USDA61, indicating that *Rj4* blocked *B. elkanii* infection in the early stages. Accumulation of H_2O_2 and salicylic acid (SA) was observed in the roots of BARC-2 inoculated with USDA61. Transcriptome analyses revealed that inoculation of USDA61, but not its T3SS mutant in BARC-2 induced defense-related genes, including those coding for hypersensitive-induced responsive protein, which act in effector-triggered immunity (ETI) in *Arabidopsis*. These findings suggest that *B. elkanii* T3SS triggers SA-mediated ETI-type response in *Rj4*-soybean, which consequently blocks symbiotic interactions. This study revealed a common molecular mechanism underlying both plant–pathogen and plant–symbiont interactions and suggests that establishment of a root nodule symbiosis requires the evasion or suppression of plant immune responses triggered by rhizobial effectors.

POSTER 8-12

Genetics and Metabolomics Analysis of a Non-efficient *Medicago*-rhizobia Symbiosis

Gonzalo Torres Tejerizo[1,2], Hanna Bednarz[1], Daniel Wibberg[1], Juliet Nilsson[2], Lucas Castellani[2], Mariano Pistorio[2], Antonio Lagares[2], Andreas Schlüter[1], Alfred Pühler[1] and Karsten Niehaus[1]

[1] *CeBiTec, Bielefeld Universität, Bielefeld, Germany*
[2] *Instituto de Biotecnología y Biología Molecular, CONICET - Universidad Nacional de La Plata, La Plata, Argentina*

Alfalfa (*Medicago sativa*) is the most widely cultivated forage legume for cattle and animal feeding, occupying about 32 million hectares over the world. Therefore, management of the N_2-fixing symbiosis is important to maximize the production of this crop. Symbiosis is a very complex process and, particularly, the *Medicago-rhizobia* symbiosis is highly specific. *Ensifer meliloti* is the model of efficient rhizobia able to infect *Medicago* that, after several steps, form nodules where they differentiate into bacteroids. These bacteroids are responsible for a successful nitrogen-fixing stage. Nevertheless, not only *Ensifer* strains are able to nodulate *Medicago*; Oregon-like strains are also able to infect alfalfa. Oregon-like rhizobia (*e.g.* LPU83) are acid tolerant, very competitive for the nodulation of alfalfa in acids soils, but inefficient for biological nitrogen fixation. The nodules developed by LPU83 harbored only few bacteroids compared to those of plants infected by the reference strain *E. meliloti* 2011 and the senescence zone develops early. This large senescence zone allows a short nitrogen fixation zone (Wegener *et al.*, 2001).

Previously, we have presented a draft genome of LPU83 (Wibberg *et al.*, 2014). In order to elucidate the deficient phenotype of LPU83, genome analyses and metabolomics were performed. Also in a previous work, we study nodulations factors and *nod* gene cluster (Torres Tejerizo *et al.*, 2011). Now, we evaluated others genes also involved in the synthesis of symbiosis determinants. Comparison of exopolysaccharide synthesis gene cluster show that while in *E. meliloti* 2011 only one gene cluster is found, two clusters were found in LPU83, one in the chromosome and one in plasmid pLPU83a. Some of the genes were duplicated or not present in the clusters, but elsewhere in the genome. Deletion of the *exo* cluster of the plasmid did not modify the nodules development. Lipopolysaccharide synthesis gene cluster were also compared and the organization among *E. meliloti* and LPU83 was found more similar than with *Rhizobium etli* or *Rhizobium leguminosarum*. Moreover, the genes for nitrogen metabolism enzymes were present in LPU83. Metabolomics analyses of the full nodules of *E. meliloti* 2011 and LPU83 were performed. Striking differences in metabolite profiles were obtained, among them dicarboxylic acids and sugar compounds.

Accordingly, Oregon-like rhizobia could be used as key reference strains to perform comparatives studies aiming to detect and identify genes that could be involved in an efficient or inefficient symbiosis.

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Wibberg, D., et al. 2014. J Biotechnol, 176, 40-1.

POSTER 8-13

PGPB *P. fluorescens* creates Oxidative Stress in *B. napus* in a Hydroponics Growth Pouch System

Aydin Enez, Lee Hudek, Wesley Webster, Dasun Premachandra, Lambert Bräu

Centre for Regional and Rural Futures, Deakin University, Melbourne, Australia

Brassica napus (canola) is the largest oilseed crop in Australia, and we have previously identified PGPB isolates that are capable of enhancing *B. napus* growth and overall yield, after stress-induced delayed germination. Four *P. fluorescens* strains isolated from *B. napus* rhizosphere samples were selected based on possession of traits known to enhance *B. napus* growth including ACC deaminase activity, phosphate solubilisation, IAA production, siderophore production, and catalase activity. *Pseudomonas fluorescens* isolate S1-27 was selected for further analysis as to its potential to reduce oxidative stress in plants grown in soil-based versus hydroponic growth systems.

Whilst *P. fluorescens* S1-27 showed to enhance *B. napus* growth in a soil-based system, it increased oxidative stress in hydroponic growth pouches, resulting in reduced *B. napus* growth. This is the first study to show significant differences in soil and hydroponics systems for PGPB inoculated *B. napus*. Using a DAB assay, quantification of total peroxidase activity for *B. napus* challenged with 800 µM H_2O_2 after 11 days, compared with *B. napus* co-inoculated with *P. fluorescens* S1-27 and stressed with H_2O_2, showed that *P. fluorescens* S1-27 peroxidase activity contributed to reducing the oxidative stress in plants by increasing total peroxidase activity in this system. Using qRT-PCR, expression of *B. napus* catalase genes (*Cat1*, *Cat2* and *Cat3*) in plants stressed with H_2O_2 in a soil-based system was reduced when co-inoculated with *P. fluorescens* S1-27 after 11 and 30 days. These findings show that whilst *P. fluorescens* S1-27 is capable of enhancing growth of *B. napus* in a soil-based system, it caused growth limiting oxidative stress in *B. napus* in a hydroponics growth pouch system.

POSTER 8-14

Selection Regime Drive Divergent Phenotypic Adaptation During the Experimental Evolution of Legume Symbionts

Marta Marchetti[1], Yasmina Yousfi[1], Camille Clerissi[1], Marc Valls[4], Delphine Capela[1], Carine Gris[1], Alain Jauneau[2,3], Catherine Masson-Boivin[1]

[1] *INRA, LIPM, Castanet Tolosan, France*
[2] *CNRS, Castanet-Tolosan, France*
[3] *UPS, Fédération de Recherches Agrobiosciences, Interactions, Biodiversity, Plateforme d'Imagerie TRI, Castanet-Tolosan, France*
[4] *Centre de Recerca Agrigenòmica (CRAG), Barcelona*

The current evolutionary scenario for the emergence of rhizobia, implies the acquisition via horizontal gene transfer (HGT) of a set of key symbiotic genes, the nodulation and nitrogen fixation genes located on mobile elements, allowing conversion of soil bacteria into facultative mutualistic symbionts of legumes under the strong selection pressure of the plant. However, transfer in itself is usually unproductive between evolutionary distant taxa. It may have involved a post-HGT adaptation step, allowing full expression of the symbiotic potential via genome reprogramming, driven by iterated selection through alternation of saprophytic-symbiotic lives [1].

To get insights on the mechanisms that drive the emergence of rhizobia, we replay the HGT-driven evolution of a new rhizobial genus by first introducing the symbiotic plasmid of the *Mimosa* symbiont *Cupriavidus taiwanensis* into the phytopathogen *Ralstonia solanacearum*, generating a non-nodulating chimera. Then, we challenged transconjugants to become *Mimosa* symbionts through serial *ex planta-in planta* passages following a short or a long regime of infection of 21 or 42 days, respectively. Evolution thought *ex planta-in planta* short cycles of 21 days resulted in acquisition of nodulation and dramatic improvement of intracellular infection of evolved clones [2,3]. Here, we report on the experimental evolution of the nodulating *Ralstonia* chimeric ancestors into *Mimosa* symbionts by using long *ex planta-in planta* cycles of 42 days, and compared the phenotype and genome evolution of short- and long-evolved clones and the impact of selection regime on the lab-evolution of *Ralstonia*.

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POSTER 8-15

The Expansion of Interface Membrane in Infected Cells of *Medicago truncatula* Root Nodules: Putative Mechanisms

Elena Fedorova[1,2], A. Gavrin A[1], V. Lara-Dampier[2] , T. Coba de la Peña[2], O. Kulikova[1], J. J. Pueyo[2], M. M. Lucas[2]

[1] *Laboratory of Molecular Biology, Department of Plant Sciences, Wageningen University, The Netherlands*

[2] *Instituto de Ciencias Agrarias, CSIC, Serrano, Madrid, Spain*

Specialized infected cells in root nodule are housing rhizobia and providing the environment for symbiotic nitrogen fixation. The rhizobia are confined to asymmetrical protrusions of modified plasma membrane forming infection threads, unwalled droplets and symbiosomes. These membrane compartments rapidly increase in number and volume due to the expanding bacteria. Plasma membrane capacity to stretch is not more than 3%(1), due to this the delivery of a new membrane vesicles is crucial for the growth of the interface membrane. We hypothesized that the membrane stretch/damage caused by expanding bacteria creates a vector for vesicle fusion to the interface membrane. To test the hypothesis that the mechanisms of membrane repair are involved we selected Ca^{2+} sensor synaptotagmin known to be operational in membrane repair in Arabidopsis (2). The sequence of A. thaliana synaptotagmin1, was used to retrieve M. truncatula homologs MtSyt1, MtSyt2 and MtSyt3. Using double silencing constructs we found that these homologs are operative in bacteria release, host cell growth and symbiosome maturation. The localization shows specific spatial and temporal pattern, MtSyt2, MtSyt3 were found on the infection threads/unwalled droplets and MtSyt1 on symbiosome membranes. We conclude that the membrane repair mechanisms are involved in the expansion of interface membrane.

The development of infected cells is accompanied by the drastic change in the volumes and the osmotic status of the endomembrane compartments (3). One of the driving forces of the expansion is a turgor pressure which depends on the uptake and the distribution of inorganic ions (4). Therefore, the vesicle traffic in infected cells must be coordinated with the change in the ion transport. To resolve the ion compartmentalization in root nodules we analysed the ionomic of root nodule using low temperature-scanning electron microscope and X-ray microanalysis. We have examined infected cells at different developmental stages and and non-infected cells of the outer and inner nodule cortex. The most pronounced dynamics was observed in the distribution of K^+ and Na^+. To detect the role of putative transport pathways involved in these changes we have studied the expression and the localization of the potassium transporters (AKT1,SKOR) and the sodium/hydrogen exchanger SOS1 in root nodules. The localization of these transporters on membranes was dependent on the developmental stage of symbiosis, suggesting the role of K^+ and Na^+ ions in the maintenance of osmotic pressure and the expansion of endomembrane compartments harboring the microsymbiont.

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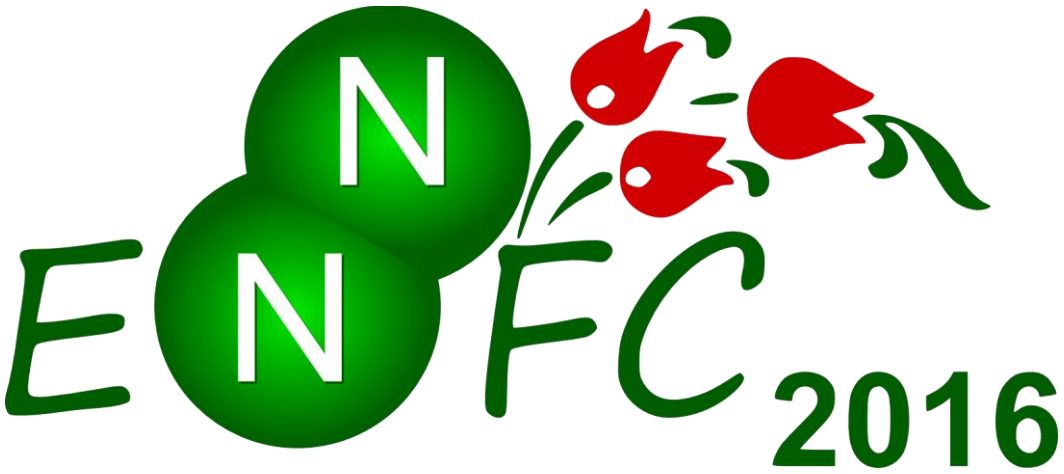

POSTER SESSION 9

Evolution, Diversity and Ecology

Room Uranus

POSTER 9-1 /LIGHTNING TALK/

Stress-induced DNA Double-strand Break NHEJ Repair in *Sinorhizobium meliloti*: A Function in Lateral Gene Transfer?

Pierre Dupuy, Laurent Sauviac, Claude Bruand

LIPM, Université de Toulouse, INRA, CNRS, Castanet-Tolosan, France

DNA double-strand breaks (DSBs) are a permanent threat for living organisms, as they are a source of genome instability. Inefficient DSBs repair can lead to disorders ranging from reduced survival in bacteria to the development of cancers in humans. DSBs are repaired by several systems, including non-homologous end-joining (NHEJ). In eukaryotes, the main NHEJ proteins, Ku70 and Ku80, bind DNA ends as a heterodimer, and then recruit several additional proteins including enzymes which catalyze the modification and ligation of DNA ends. NHEJ has also been characterized in a limited number of bacteria, where the repair mechanism appears to be less complex than in eukaryotes. Indeed, only two proteins are required: a homodimeric Ku protein, and a multifunctional LigD enzyme which modifies and ligates the DNA ends. However, most studies were performed on bacterial species encoding a single ku-ligD pair. Actually, many bacterial species encode multiple copies of these genes, whose relative contributions to NHEJ in vivo are so far unknown.

The Sinorhizobium meliloti genome encodes four putative Ku (ku1-4) and four putative LigD (ligD1-4). To date, a single study conducted on this model bacterium showed that every ku single mutant is more sensitive than the wild type strain to ionizing radiations (1) showing that all ku genes are involved in NHEJ repair of DSBs in this organism.

Here, using a plasmid circularization assay, we performed a comprehensive genetic characterization of NHEJ repair in S. meliloti, and clarified the respective contributions of the various ku and ligD genes. We also demonstrated that NHEJ repair is activated under various stress conditions, including heat and nutrient starvation, and that part of this repair is under the control of the general stress response regulator RpoE2. Finally, for the first time in bacteria, we provided evidence that NHEJ not only repairs DSBs, but can also erroneously integrate heterologous DNA molecules into the breaks.

Altogether, our data provide new insights into the mechanisms of DSB repair in bacteria which encode multiple NHEJ systems, but also suggest that NHEJ might contribute to the evolution of bacterial genomes under adverse environmental conditions by participating in the acquisition of foreign DNA from distantly related organisms during horizontal gene transfer events.

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POSTER 9-2 /LIGHTNING TALK/

Mixed Nodules in *Sinorhizobium meliloti* - *Medicago sativa* Symbiosis Suggest the Presence of a Cheating Behavior

Alice Checcucci[1], Elisa Azzarello[2], Marco Bazzicalupo[1], Marco Galardini[3], Alessandra Lagomarsino[4], Stefano Mancuso[2], Lucia Marti[2], Maria C. Marzano[2], Stefano Mocali[4], Andrea Squartini[5], Marina Zanardo[5], Alessio Mengoni[1]

[1] *Department of Biology, University of Florence, Florence, Italy*
[2] *Department of Agri-food Production and Environmental Science (DISPAA), University of Florence, Florence, Italy*
[3] *EMBL-EBI, Wellcome Trust Genome Campus, Cambridge, United Kingdom*
[4] *Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Centro di Ricerca per l'Agrobiologia e la Pedologia (CREA-ABP), Firenze, Italy*
[5] *Department of Agronomy, Food, Natural Resources, Animals and the Environment, University of Padua, Legnaro, Padova, Italy*

In the symbiosis between rhizobia and leguminous plants host plants can form symbiotic root nodules with multiple rhizobial strains, potentially showing different symbiotic performances in nitrogen fixation. Here, we investigated the presence of mixed nodules, containing rhizobia with different levels of mutualisms, and evaluate their relative fitness in the *Sinorhizobium meliloti* - *Medicago sativa* model symbiosis.

We used three *S. meliloti* strains, the mutualist strains Rm1021 and BL225C and the non-mutualist one AK83. We performed competition experiments involving both *in vitro* and *in vivo* symbiotic assays with *M. sativa* host plants.

We show the occurrence of a high number (from 27% to 100%) of mixed nodules with no negative effect on both nitrogen fixation and plant growth. The estimation of the relative fitness as non-mutualist/mutualist ratios in single nodules shows that in some nodules the non-mutualist strain efficiently colonized root nodules along with the mutualist ones.

In conclusion, we can support the hypothesis that in *S. meliloti* – *M. sativa* symbiosis mixed nodules are formed and allow non-mutualist or less-mutualist bacterial partners to be less or not sanctioned by the host plant, hence allowing a potential form of cheating behavior to be present in the nitrogen-fixing symbiosis.

POSTER 9-3 /LIGHTNING TALK/

Structure and Functional Design of the Plasmid Regions Harboring *sym* Genes in *Rhizobium leguminosarum*: New Evidence for Intensification of Horizontal Gene Transfer and Narrowing the Host Range in Rhizobia Evolution

Elizaveta R. Chirak, V. V. Kopat, A. K. Kimeklis, E. E. Andronov, N. A. Provorov

ARRIAM, St-Petersburg, Russia

Our research is aimed to elucidate the trade-off between the structure of *sym* gene clusters in the evolutionary advanced rhizobia (*Rhizobium leguminosarum*), their host specificity and the impact of horizontal gene transfer (HGT) in the rhizobia evolution.

We investigated the structure of plasmid loci containing *sym* genes (*nod*-, *nif*- and *fix*-operons) in *Rhizobium leguminosarum* strain Vaf12 obtained from nodules of *Vavilovia formosa* (the relict legume, close to a common ancestor of the *Fabeae* tribe) in comparison to 7 strains of *R. leguminosarum* differing in origin and host specificity, including 4 strains of biovar *viciae* - symbionts of pea (TOM, UPM1131, 3841) and forage beans (248), as well as 3 strains of biovar *trifolii* - clover symbionts (WSM1689, WSM1325, SRDI943). We shown that the strains of *R. leguminosarum* bv. *viciae*, having *nod*X gene (controls acetylation of Nod-factor that determines the ability of rhizobia to establish symbiosis with broad spectrum of host plants, including the "Afghan" pea lines homozygous for sym^{2A} allele) are characterized by a less compact clustering of *sym*-genes than strains lacking *nod*X. In strains with *nod*X, symbiotic cluster size is 94.5±3.5 kb, while the proportion of *sym*-genes in this cluster is 36.5±1.5%. For the strains without *nod*X these indices are 61.7±3.7 kb and 56.3±1.4%, respectively (significant difference at P_0 <0.01). No correlation between the *sym* gene cluster size and the presence of *nodX* was found in biovar *trifolii* strains for which *nodX* is not involved in the host specificity control.

We detected the synthenic structures of *sym* genes in strains Vaf12, UPM1131 and TOM, and synthenic structures of areas located between *sym* genes in strains Vaf12, TOM and WSM1689. The correlation coefficients between the matrices of genetic distances in the analyzed strains for *nod*ABC, *nif*HDK, *fix*ABC operons, reach 0,993±0,002, while for plasmid sites located between *sym*-genes the correlations are substantially less (0.706±0.010). In these inter-*sym*-gene areas, 21-27% of genes are involved in the transport and metabolism of amino acids, which is substantially greater than the average for the genome of *R. leguminosarum* bv. *viciae* (11-12%).

These data suggest that the evolution of *R. leguminosarum* bv. *viciae*, defined by a narrowing of the host range (associated with the loss of *nod*X), was accompanied by a reduction of the *Sym* plasmid areas, located between *sym*-genes, as well as by specialization of these areas to perform functions related to symbiotic N_2 fixation. The observed increase of *sym*-gene density may be associated with the intensification of HGT in the populations of rhizobia, which determines the speed of evolution for the symbiotic gene system.

POSTER 9-4 /LIGHTNING TALK/

The Range of Rhizobia in New Zealand Soils

Mitchell Andrews

Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln, Christchurch, New Zealand

Most legume species can obtain a substantial amount of their nitrogen (N) requirements for growth from symbiotic bacteria (general term rhizobia) in root nodules and this can give them an advantage in low N soils if other factors are favourable for growth. There are only four genera of native legumes on the main New Zealand islands. These are *Carmichaelia, Clianthus, Montigena* and *Sophora*. However, over the past 150 years, several legumes have become important crop plants in New Zealand agricultural systems. Also, over 100 legume species from different continents have become naturalised in New Zealand and several of these are now important weeds.

Recent work which genotypically characterised rhizobia of native, crop and weed legumes in New Zealand and examined their cross-nodulation ability, was reviewed. The New Zealand native legumes were exclusively effectively nodulated by novel strains of *Mesorhizobium* which did not nodulate crop or weed legumes (1, 2). Seven groups of these strains have been formally described as new species (3, 4). Clovers (*Trifolium* spp.), lucerne (*Medicago sativa*), *Lotus* spp. and grain legumes were effectively nodulated by different genera, species and biovars of rhizobia primarily originating from inoculum. Weed legumes were effectively nodulated by different genera and species of rhizobia depending on plant species (5, 6). Novel strains of *Bradyrhizobium* that cross-nodulate lupins (*Lupinus* spp.), gorse (*Ulex europeus*), European broom (*Cytisus scoparius*) and tagasaste (*Cytisus proliferus*) are widespread in New Zealand.

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POSTER 9-5 /LIGHTNING TALK/

Genetic Evidence that Local Legume Sanctions Drive the Emergence of Symbiotic Nitrogen Fixation

Delphine Capela, Benoit Daubech, Ginaini Doin de Moura, Marta Marchetti, Catherine Boivin-Masson

LIPM, Université de Toulouse, INRA, CNRS, Castanet-Tolosan, France

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The selective forces allowing the emergence and maintenance of nitrogen-fixing mutualism between legumes and rhizobia are still highly debated. The most widely accepted explanation for the evolutionary persistence of mutualism is that plants differently affect the fitness of rhizobia in nodules based of their nitrogen-fixing abilities. This "sanction" hypothesis has been demonstrated by manipulating N2 gas abundance around nodules, simulating rhizobia with different fixation efficiencies. A genetic reappraisal of this question was needed to definitely confirm the sanction hypothesis and identify experimental conditions favoring the fixation of emerging Fix+ mutations within a non fixing bacterial population.

To evaluate whether, when and how plants differently treat fixing and non-fixing symbionts, we inoculated *Mimosa pudica* with either its wild-type symbiont *C. taiwanensis* or its isogenic *nifH* deletion mutant, or with a mix of both strains, and monitored the reproductive fate and bacteroid persistence of nodule bacteria along time.

We confirmed that plant sanctions non-fixing bacteria at the nodule level. Experimental evolution showed that legume sanctions drive the fixation of emerging nitrogen-fixing symbionts within a non-fixer population.

POSTER 9-6 /LIGHTNING TALK/

Symbiotic Divergence of *Rhizobium leguminosarum* Strains from Relict Legume *Vavilovia formosa*: A Background for Identification of Novel Biovar

Anastasiia K. Kimeklis[1], V. I. Safronova[1], A. A. Belimov[1], E. E. Andronov[1], I. G. Kuznetsova[1], A. L. Sazanova[1], A. R. Pukhaev[2], K. P. Popov[3], J. A. Akopian[4], N.A. Provorov[1]

[1] *All-Russia Research Institute for Agricultural Microbiology, St.-Petersburg, Russia*
[2] *Gorsky State Agrarian University, Vladikavkaz, Russia*
[3] *North-Ossetian Nature Reserve, Republic North-Ossetia-Alania, Alagir, Russia*
[4] *Institute of Botany of National Academy of Sciences of Republic Armenia, Yerevan, Republic of Armenia*

Tribe *Fabeae* (syn. *Vicieae*) comprises 5 genera, and typical rhizobial symbiont of plants from this tribe is *Rhizobium leguminosarum* bv. *viciae* (1). Symbiosis between this genus of rhizobia and plants of *Fabeae* tribe playing a major part in agriculture ecosystems, it's specifity is thoroughly studied (2). Exception is relict wild-growing *Vavilovia*, whose genus consists of single species *Vavilovia formosa*. It is believed, according to both comparative morphology and classical genetics, to be the closest relative to the extinct common ancestor of tribe *Fabeae* (3). Study of *Vavilovia*'s symbiosis is complicated by its low distribution of populations and specific inaccessible environment of the high-mountain Caucasian region.

In this study we undertook expeditions to North Ossetia, Dagestan and Armenia, where populations of *Vavilovia* were found and several plants with nodules were collected.

For the genotypic characterization of 20 *Rhizobium*-related isolates full gene of 16S rDNA and symbiotic genes from *nod* and *nif* operons were sequenced. On the basis of chromosomal background, phylogenetic analysis showed that isolated strains are the closest to *Rhizobium leguminosarum* bv. *viciae*. They formed several clusters, which didn't correlate with geographic distribution. Phylogenetic study of symbiotic background demonstrates that isolates form separate cluster inside *R. leguminosarum* bv. *viciae* clade. It was also revealed that all 20 *R. leguminosarum* strains are carriers of *nod*X gene, encoding O-acetyl transferase, a nod-factor decorator, which is essential for forming of symbiosis with *Pisum sativum* cv. *Afghanistan*. All strains fall into one group on the basis of *nod*X sequence.

Sterile tube-test experiments with strains representing different geographic regions were conducted using three host plants – *P. sativum* SGE, *P. sativum* cv. *Afghanistan* and *Vavilovia formosa*. These tests demonstrated that isolates formed nodules on all plants, although nodules had different ability to fix nitrogen. Nitrogen-fixing activity was detected mostly only in symbiosis with *Vavilovia formosa* plants, although some strains formed nod^+fix^+ phenotype on *P. sativum* cv. *Afghanistan*.

Genome sequencing of the "true" Vavilovia symbiotic isolate Vaf12 demonstrated one notable feature: the spacious arrangement of sym-genes. Broad host-range strains Vaf12 and TOM, both of which contain *nod*X, have more extended sym-regions than strains without *nod*X. It hints, that loss of some sym-genes (nodX, in particular) and compaction of sym-region may be the stages in process of host specificity constriction in *R.leguminosarum*.

This work is supported by RSF grant 14-26-00094.

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POSTER 9-7 /LIGHTNING TALK/

The Impact of Host Genotype and Geographical Origin on *Rhizobium leguminosarum* Genetic Diversity

Sara Moeskjær[1], Asger Bachmann[1], Bjarni Vilhjalmsson[2], Luc Janns[1], Mikkel H. Schierup[2], J. Peter W. Young[3], Stig U. Andersen[1]

[1] *Department for Molecular Biology and Genetics, Aarhus Universitet, Denmark*
[2] *Bioinformatics Research Center, Aarhus University, Denmark*
[3] *University of York, York, United Kingdom*

The aim of the NCHAIN project (http://mbg.au.dk/nchain) is to increase the yield of organic clover fields by analyzing the genetics of both clover plants and their rhizobium symbionts. To initiate the study of rhizobium genomics, 249 rhizobium strains were isolated from white clover nodules from plants grown in France (74), the United Kingdom (33), and Denmark (142). For each site the clover varieties are known, and representative soil samples were collected and sent for chemical analysis. Furthermore site-specific GIS data was collected based on the site coordinates.

The genetic relatedness of the rhizobium strains was assessed through analysis of phylogenetic trees. The core genes *recA* and *rpoB*, are clearly divided into different clades corresponding to genospecies of *R. leguminosarum* as previously described (1). In contrast, phylogenies of the symbiosis genes *nodA* and *nodD*, located on the pRL10 plasmid, were grouped primarily according to geographic origin. To elaborate on these findings we are carrying out a Quantitative Qualitative Amplicon Diversity (QQAD) analysis on nodule (n>100) and soil samples from each of the 170 sampled sites to study the diversity of rhizobia within and between each site. Furthermore, comparisons of soil and nodule populations will reveal the effects of selection by the clover host.

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POSTER 9-8 /LIGHTNING TALK/

Evolution of *fixNOQP* Genes Encoding for the High-affinity Cytochromoxidase: Insight from the Genomes of Symbionts from the Relic Legume *Vavilovia formosa*

Vladimir V. Kopat, E. E. Andronov, N. A. Provorov

ARRIAM, St-Petersburg, Russia

Recently, our Caucasian expedition obtained the relict legume plant *Vavilovia formosa* with well sampled roots and intact root nodules, suitable for rhizobia isolation (the first successful isolation). It is curious, that *Vavilovia formosa* as the closest living relative of the last common ancestor of the *Fabeae* tribe (1) can be considered as a valuable source of information about rhizobia evolution. Specifically, broad variety of microorganisms including *Rhizoboium, Tardiphaga* and *Bosea* was isolated from the nodules of vavilovia (2). The genome sequencing of these isolates and close inspection of this data led us to the earliest events of nodule bacteria evolution (*sym*-gene acquisition and *sym*-cluster formation).

In the context of evolution signs in rhizobia genomes, *Tardiphaga* Vaf07 is the most interesting strain (taxonomically close to *Rhodopseudomonas*). There are *fixABGHISKNOPQ* genes into *fixAB*, *fixGHSNOQPK* and *fixISNOQP* clusters, but lack of crucial *nod* and *nif* genes in the Vaf07 genome (that is the reason of inability to form nodules under gnotobiotic conditions). In spate of lacking *nod* genes, *Tardiphaga* is regularly isolated from nodules of Vavilovia and some other legumes, possibly due to co-infection with *nod*-harboring strains (3). As can be seen, *Tardiphaga* has two copies of *fixNOQP* cluster, coding a high affinity cbb3-type cytochrome c oxidase.

The most intriguing issue about these two copies is their deep phylogenetic divergence: the first copy belongs to the *Bradyrhizobiaceae* cluster while the second one belongs to the *Sinorhizobium-Rhizobium* cluster. According with our suggestion, the first step of rhizobium-legume symbiosis formation was acquisition of *fix* genes by rhizobia, so it is reasonable to evaluate significance of *fix* genes in the rhizobia evolution.

After a detailed analysis of *fixNOQP* genes polymorphism among different rhizobia and strains from diverse groups of *Proteobacteria* we concluded that evolution of these genes includes at least two duplications with subsequent divergence of copies. *Tardiphaga* Vaf07 seems to be the closest relative of ancestor strain in which the first duplication occurred. Considering, that the proposed ancestor of *Rhizobium* (*Agrobacterium*) doesn't have *fix* genes at all, it can be assumed that tardiphaga's ancestor was an origin of *fix* genes for *Rhizobium* and the transfer of these genes was occurred under conditions close to vavilovia symbiosis. The revealed *fixNOQP* polymorphism may reflect to adaptation of the *Rhizobiales* to various anaerobic niches occupied in soil and in plant tissues.

This finding highlights the interplay of vertical transmission and horizontal gene transfer in the rhizobia evolution and the importance of functional diversification of different copies of *fix* genes in diversification of the modern rhizobia species.

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POSTER 9-9 /LIGHTNING TALK/

Carbon Utilisation by Strains of *Rhizobium* spp. in Sterile Soil

Kathryn Wigley[1], Steve Wakelin[2], Derrick Moot[1], Hayley Ridgway[1]

[1] *Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln, Canterbury, New Zealand*
[2] *AgResearch Ltd, Lincoln Research Centre, Lincoln, Canterbury, New Zealand*

In New Zealand, the bacterium *Rhizobium leguminosarum* bv. *trifolii* strain TA1 is used to commercially inoculate white clover seed. Recently, the need for inoculating white clover in New Zealand has been questioned. This is due to the inability of TA1 to deliver plant growth benefits because it cannot compete with high titres of naturalised rhizobia in the soil (1). However, naturalised strains have variable symbiotic potential compared with TA1 ranging from 0 – 170% (1). Effective naturalised strains adapted to New Zealand soils could be the key to improving commercial inoculants which are greater than 60 years old (2).

Rhizobium strains that show promise *in vitro*, often fail to perform in the field. A critical reason is lack of understanding of the interactions of the isolates within the soil environment (3). γ MicroResp™ is a novel modification of the 96-well based MicoResp™ system (4) which uses γ-irradiated soil. It allows the measurement of a microorganism's ability to utilize common C sources released in rhizosphere exudates within a physical soil background. This provides fundamental information on a strains free-living saprophytic ability.

For this study, 19 diverse rhizobia strains sourced from an international collection and 9 strains recovered from soils in Canterbury, New Zealand, were tested for their ability to utilise 14 carbon sources. The carbon sources were predominantly sugars and amino acids commonly found in the rhizosphere.

The international strains of rhizobia formed 9 distinct phenotypic groups (p<0.05) and the New Zealand strains formed four distinct phenotypic groups (p<0.05) based on differences in soil C-utilization. Variation in carbon utilization among the 19 international strains could not be attributed to geographic origin. In both the international and New Zealand collections, some groups of strains utilised a wider variety of carbon compounds to a greater degree compared with strains in other groups. The ability to use a broad range of C sources provides information about the ability of a strain to exist saprophytically in the rhizosphere (5). This knowledge will aid in improved selection and deployment of "environmentally fit" commercial inoculants.

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POSTER 9-10

1-aminocyclopropane-1-carboxylate Deaminase Gene Correlates with Symbiotic Lineages Nodulating *Cicer canariense*

Juan Pérez-Yépez[1], J. Donate-Correa[2], M. León-Barrios[1]

[1] *Departamento de Bioquímica, Microbiología, Biología celular y Genética, Spain*
[2] *Unidad de Investigación Hospital Universitario Nuestra Señora de Candelaria, Spain*

The 1-aminocyclopropane-1-carboxylate deaminase (ACCd) is an enzyme that can promotes plant growth under different stress conditions by decreasing the levels of 'stress ethylene' (1-2).

Cicer canariense is a wild chickpea endemic to La Palma (Canary Islands, Spain). In its natural habitats this legume establishes an efficient nitrogen fixing symbiosis with mesorhizobial strains belonging to different species and three symbiovars (3). In this work, 84 strains were tested by the presence of the gene codifying the ACCd (*acdS*). It was found that at least 57% of these mesorhizobia had this gene. However, none of them showed any ACCd activity in vitro assays (bacteria growing in a minimal medium with ACC as a sole nitrogen and carbon source). A phylogeny based on *acdS* gene sequences showed correlation with the nodulation (*nodC* and *nodA*) and fixation (*nifH*) genes for strains from a novel symbiovar, the symbiovar ciceri and the type strain of *M. loti* NZP2213T (also an effective symbiont of *C. canariense*), while a third group of strains within the symbiovar loti clade were dispersed in three subbranches. RNA from mature nodules (8-10 weeks old) was used to assess the *acdS* gene expression. Our results showed an expression of *acdS* gene under symbiotic conditions; furthermore, we observed different levels of expression depending on the symbiotype. Results here presented suggest that, as it has been shown for other mesorhizobial species (4), the *acdS* gene can be localized in the symbiotic island.

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POSTER 9-11

A Phylogenomic Approach to Unravel the Evolution of the Nitrogen-fixing Root Nodule Symbiosis

Maximilian Griesmann[1], J. M. Ané[2], A. Berry[3], G. Haberer[4], M. Spannagl[4], S. Cheng[5], V. Hocher[6], L. Wall[7], P. Normand[8], K. Pawlowski[9], B. Billault-Penneteau[1], R. Geurts[10], G. Werner[11], K. Mayer[4], G. Oldroyd[12], P. M. Delaux[13], M. Parniske[1]

[1] *Faculty of Biology, University of Munich (LMU), Germany*
[2] *Laboratory of Genetics, University of Wisconsin-Madison, United States*
[3] *Department of Plant Sciences, UC Davis, United States*
[4] *PGSB, Helmholtzzentrum München, Germany*
[5] *BGI, China*
[6] *Laboratory of Tropical and Mediterranean Symbiosis, Institute of Research for Development, France*
[7] *Department of Science and Technology, University of Quilmes, Argentina*
[8] *Ecologie Microbienne, University of Lyon, France*
[9] *Department of Ecology, Environment and Plant Sciences, Stockholm University, Sweden*
[10] *Laboratory for Molecular Biology, University of Wageningen, The Netherlands*
[11] *Department of Ecological Science, University of Amsterdam, The Netherlands*
[12] *Department of Cell & Developmental Biology, John Innes Centre, United Kingdom*
[13] *LRSV, University of Toulouse, France*
m.griesmann@bio.lmu.de

The Root Nodule Symbiosis (RNS) can be only established by species within a monophyletic clade consisting of the four orders Fabales, Fagales, Rosales and Cucurbitales (FaFaCuRo). However, among these orders the frequency of symbiotic plants, nodule morphology and the bacterial symbionts differ greatly. A parsimonious hypothesis claims that multiple independent gains of the RNS followed a single genetic predisposition event in the common ancestor of the FaFaCuRo (1). To date the genetic nature of this event is unclear. Given this hypothesis is true, the symbiotic plants are likely to share genetic elements across the FaFaCuRo. Based on the probabilistic model published by Werner et al. (2), we have grouped species of the FaFaCuRo into the different symbiotic states "predisposed", "predisposition lost", and "nodulating". To identify symbiosis-specific orthologs sharing a defined presence/absence pattern across the different symbiotic states, we use a phylogenomic comparison pipeline. Until now an approach of this scale was not possible due to the lack of sequenced genomes representing the different symbiotic states. To fill these gaps, we have chosen species dispersed across the FaFaCuRo and from different symbiotic states for sequencing their genome and/or their root transcriptome. With this approach we aim to unravel the evolutionary history involved in both the legume and actinorhizal RNS and possibly part of the genetic predispostion event.

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POSTER 9-12

Beneficial Endophytic Bacteria of Pea *(Pisum sativum L.)*

Gulnar Akhtemova[1], Alexey Afonin[1,2], Ekaterina Vasilyeva[2], Vladimir Zhukov[1], Alexey Borisov[1], Igor Tikhonovich[1]

[1] *Federal State Budget Scientific Institution All-Russia Research Institute for Agricultural Microbiology, Saint-Petersburg-Pushkin, Russia*
[2] *Saint Petersburg State University, Russia*

Leguminous plants are able to form beneficial plant-microbe symbioses with microorganisms inhabiting the internal environment of the plants (endosphere): nodule bacteria, arbuscular-mycorrhizal fungi and plant-growth promoting bacteria (PGPB). Endophytic PGPB colonizing the intercellular space in various plant tissues can be isolated from all plant parts, including dry seeds. Every single type of plant is the host for one or more species of endophytic bacteria.

Aim of this work: to isolate and study culturable bacterial endophytes of pea (*Pisum sativum L.*).

Endophytic bacteria were isolated from the stems, leaves and seeds of pea (Pisum sativum L.) commercial cultivar "Triumph", which is characterized by high symbiotic potential, i.e. improved efficiency of symbiotic interactions with nodule bacteria and arbuscular-mycorrhizal fungi.

Plant organs were sterilized with 70% ethanol and 5% sodium hypochlorite, homogenized and plated on Petri dishes with agar nutrient medium TSA or 1/20 TSA.

In total, 145 strains of endophytic bacteria were isolated. Agronomically-beneficial traits (plant growth promoting activity, production of auxin-like compounds, fungicidal and antibiotic activity) were found in 18 strains. Molecular identification of these strains was performed by sequencing of V1-V9 variable regions of 16S rDNA amplified with universal primers.

Endophytic bacteria found in pea belong to 8 genera: Bacillus sp. (majority of species), Stenotrophomonas sp., Microbacterium sp., Paenibacillus sp., Kocuria sp., Staphylococcus sp., Micrococcus sp., Actinobacterium sp.

Thus, the analysis of pea tissues revealed the presence of beneficial endophytic bacteria and showed their ability to grow on synthetic growth media. Bacteria from the genus Bacillus sp. were dominant in the pea endomicrobiome, and the endophytes isolated from pea stems are considered as the most agriculturally beneficial ones, based on the assays performed.

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POSTER 9-13

Biodiversity and Selection of Indigenous Rhizobia Associated with Pea (*Pisum sativum* L.) in Soils of Western Herzegovina

Višnja Vasilj[1], Mihaela Blažinkov[2], Adrijana Filipović[1], Sanja Sikora[3]

[1] *Faculty of Agriculture and Food Technology University of Mostar, Croatia*
[2] *College of Slavonski Brod, Agricultural Department, Croatia*
[3] *University of Zagreb Faculty of Agriculture, Department of Microbiology, Croatia*

An essential element of agricultural sustainability is the effective management of nitrogen in the environment. This usually involves at least some use of biologically fixed nitrogen because N from this source is used directly by the plant, and therefore is less susceptible to volatilization, denitrification and leaching. Despite that, legume inoculation with selected, high-quality rhizobial strains has not become usual procedure in the production of these important crops in Herzegovina. Selection of the most suitable strains represent one of the main presumptions for successful inoculation due to the fact that rhizobial strains strongly differ in their effectiveness, competitiveness and compatibility (1,2). However, the presence of adapted and competitive indigenous rhizobia in soil can reduce the inoculation response even with the highly efficient commercial strains. Thus the composition and characteristics of rhizobial field populations are of great agricultural importance in the legume production (3,4). Pea (*Pisum sativum* L.) is widely cultivated plant in the area of western Herzegovina due to their favorable nutrient composition and other health benefits that were recognized more recently. The main objective of the present study was to get insight into actual composition of indigenous population of pea rhizobia and to characterize indigenous strains isolated from different soils in western Herzegovina. The indigenous rhizobial strains were isolated from pea nodules collected from different field sites in western Herzegovina. RAPD and ERIC-PCR methods were used for strain identification and evaluation of genetic diversity among indigenous rhizobia. In order to determine the nodulation ability and symbiotic efficiency of indigenous *R. leguminosarum* strains, two-year field trials were set up at different locations near Mostar. At various locations in western Herzegovina, the presence of indigenous *R. leguminosarum* strains was determined as well as the considerable level of genetic diversity within natural populations. In comparison with reference strain and non-inoculated control, most of indigenous rhizobial strains showed significantly higher nodulation ability and symbiotic efficiency. The application of two indigenous strains (V1 and V13) isolated from soils in the area of Mostar and Široki Brijeg, resulted in the most abundant nodulation and the highest symbiotic efficiency. These are the first studies of indigenous pea rhizobia in Herzegovina. The obtained data clearly confirm the importance of rhizobial strain selection and can contribute to a better understanding of rhizobial ecology.

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POSTER 9-14

Cowpea Bradyrhizobia from Coastal and Eastern Kenyan Soils are Diverse as Revealed by Proteomic and Genomic Characterization

Samuel M. Ndungu[1, 3], C. Thonar[2], M. Messmer[2], B. Vanlauwe[3] and E. Frossard[1]

[1] *ETH Zürich Plant Nutrition Group, Lindau, Switzerland*
[2] *FiBL, Research Institute for Organic Agriculture, Frick, Switzerland*
[3] *International Institute of Tropical Agriculture (IITA) c/o ICIPE Campus. Nairobi, Kenya*
Samuel.ndungu@usys.ethz.ch, mathusn@gmail.com

Cowpea yields for smallholder farmers in two agro-ecologies [Kilifi (Coastal lowland) and Mbeere (Lower midland)] in Kenya where cowpea is prevalent, could be improved through inoculation with effective and competitive Bradyrhizobia strains.

Little is known on the diversity of cowpea Bradyrhizobia in these agro-ecologies.

This study aimed at characterization (proteomic and genomic approaches) of indigenous isolates in both agro-ecologies from sites characterized by diverse soil types and land management practices.

Matrix Assisted Laser Desorption/ionization Time of Flight (MALDI-TOF) mass spectrometry (MS) based on cell lysis to release intracellular proteins, which were ionized and separated according to their mass to charge ratio. Protein masses were recorded as distinct peaks that together form a complex spectrum or fingerprint, characteristic to particular bacterial strains or species.

From nodules obtained in farmers' fields with cowpea and sites with no history of crop cultivation (nodules from trap cultures with soil inocula) 202 strains were characterized by MALDI-TOF. Of the 202 characterized isolates, 172 were identified as belonging to the genus *Bradyrhizobium*, clustering in seven distinct similarity groups. Besides *Bradyrhizobium, Rhizobium sp.*, *Rhizobium radiobacter*, *Enterobacter cloacae* and *Staphylococcus warneri* were identified and 9 isolates remained unknown.

After MALDI-TOF analysis, 24 most distinct strains were selected based on; clustering showing their similarity and consideration from principle component analysis (PCA) showing the differences between strains.

Polymerase chain reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of the 16S-23S rDNA intergenic spacer region (IGS) on 24 revealed 3 genetic profiles, IGS types I, II and III. From ongoing genomic characterization, preliminary results from phylogenetic analysis of 16S rRNA gene sequences showed that they belong to the genus, *Bradyrhizobium*.

Ultimately, we reveal that based on proteomic and genomic approaches strains are diverse and further, Principal component analysis will be used to link different land practices and various soil properties from sites in different agro-ecologies to Bradyrhizobia diversity.

POSTER 9-15

Cultivation of Plant-associated Bacteria Belonging to the Phylum *Verrucomicrobia*

Wiebke Bünger, Jana Müller, Thomas Hurek, Barbara Reinhold-Hurek

Institute of Microbe-Plant Interactions, Faculty of Biology and Chemistry, University Bremen, Bremen, Germany

Cultivation–based studies of microorganisms living in association with plants are essential and important tools in understanding the roles and functions of these organisms in their natural niche. During this study bacteria were isolated from the rhizoplane as well as the endosphere of rice roots. Along the way, the efficiency of root- surface sterilization for cultivation-independent approaches was evaluated. We found that a surface sterilization by the commonly used ultrasonication is not sufficient to remove the majority of surface bacteria. In contrast, a chemical treatment with sodium hypochlorite was highly efficient (1).

By cultivation-dependent approaches, a broad phylogenetic range of about 100 different bacterial isolates were brought to pure culture. Amongst other bacterial phyla, four putatively novel species of the phylum *Verrucomicrobia* were isolated from rice roots and further characterized. Members of this phylum are widely distributed, rather abundant in soils and highly diverse. This phylum can be divided into 7 subdivisions, consisting of cultured and uncultured strains (2). A phylogenetic assignment by sequencing of the partial 16S rDNA sequences showed that three of our strains could be classified to subdivision 2 and subdivision 4, whereas the fourth strain could not be confidently assigned to any subgroup because of high phylogenetic distance.

So far, there is a lack of knowledge about the interaction of *Verrucomicrobia* with plants due to low cultivation rates and consequently a low number of cultivated strains. Only few studies so far dealing with the rhizosphere of leek and potato described the abundance and interaction of *Verrucomicrobia* subdivision 1 with plants (3+4).

Thus, the four novel strains were characterized with respect to major characteristics like plant growth promoting capabilities, growth, and morphology. Inoculation of rice seedlings in gnotobiotic culture followed by fluorescence microscopy showed that the bacteria formed tight biofilms along the roots without influencing plant growth negatively, however they varied in colonization density. Some strains showed the capability to produce indole-acetic acid and to solubilize phosphate, as putatively plant growth-promoting characteristics. Hence, our strains may help to get new insight into this yet sparsely characterized bacterial phylum.

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POSTER 9-16

Design of Plant Probiotic Consortia of *Rhizobium* and Endophytic Bacteria for Application to Legume and Cereal Crops in Lanzarote (Canary Islands)

Esther Menéndez[1], Jose D. Flores-Félix[1], Marta-Helena Ramírez-Bahena[2,3], Juan Pérez-Yepes[4], Fernando Sánchez-Juanes[5], Jose Manuel González-Buitrago[5], Milagros León-Barrios[4], Alvaro Peix[2,3], Encarna Velázquez[1,3]

[1] *Departamento de Microbiología y Genética, Universidad de Salamanca, Salamanca, Spain*
[2] *IRNASA-CSIC, Salamanca, Spain*
[3] *Unidad Asociada Universidad de Salamanca-CSIC 'Interacción Planta-Microorganismo'. Salamanca. Spain*
[4] *Departamento de Bioquimica, Microbiología, Biología Celular y Genética, Universidad de La Laguna, Tenerife, Spain*
[5] *Instituto de Investigación Biomédica de Salamanca (IBSAL) Salamanca, Spain*

Increasing food production and limiting environmental pollution are the main concerns in the current scenario of continuous human population growth in the World. One of the most reliable alternative of chemical fertilizers for a sustainable agriculture is the application of biofertilizers based on plant probiotic bacteria, which boost plant development through several plant growth promotion mechanisms (1). From all bacteria usually associated with plants, cultivable plant endophytes able to promote the plant growth are the most promising plant probiotics for different crops (2). Within them, legume nodule and cereal root endophytes, have been analysed in some works focusing on their ability to promote the plant growth *in vitro,* but there are few studies including plant assays (3).

In this study, we analysed the diversity of cultivable endophytic bacteria isolated from pea nodules and maize roots cultivated in a soil from Lanzarote (Canary Islands). These strains were analysed by MALDI-TOF MS, which mainly allowed the identification of human, animal and plant pathogens and by 16S rRNA gene sequencing. We analysed the *in vitro* plant growth promotion potential of the isolated strains based on their ability to movilize nutrients and to produce phytohormons and siderophores.

Our results showed the presence in pea nodules of several species from genera *Bacillus* and *Paenibacillus*, two genera also present in maize roots. Nevertheless, some strains belonging to pathogenic species from genera *Pantoea, Enterobacter, Rahnella* or *Pseudomonas* were also present in these roots. These results showed the need of a correct identification of bacterial endophytes before selecting strains for biofertilizers design. We selected *Rhizobium* and non-pathogenic endophytic strains presenting different *in vitro* plant growth mechanisms for plant experiments, which showed the ability to promote pea and maize plant growth. *Rhizobium* and maize endophytic strains consortia were also tested in plants, showing that they are good plant probiotics for both pea and maize and can be used to replace all or part of chemical fertilizers in greening practices, contributing to the sustainability of agriculture.

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POSTER 9-17

Diversity and Genome Analysis of Novel Nitrogen Fixing Microsymbionts Associated with Legumes in Two Contrasting Climatic Regions of India

Nisha Tak[1], Archana Ojha[2], Bhawana Chouhan[1], Garima Bissa[1], Satyawada R. Rao[2], Saroj K. Barik[3] and Hukam S. Gehlot[1,3]

[1] *BNF and Microbial Genomics Lab., Department of Botany, Jai Narain Vyas University, Jodhpur, Rajasthan, India*
[2] *Department of Biotechnology and Bioinformatics, North-Eastern Hill University, Shillong, Meghalaya, India*
[3] *Department of Botany, North-Eastern Hill University, Shillong, Meghalaya, India*

The diverse climatic regions of India due to its wide range of latitudinal, longitudinal and altitudinal variations create numerous ecological niches that offer conducive environment for coevolution of legumes and associated microsymbionts. However, the diversity of these native microsymbionts has not been adequately explored. In this paper, the diversity of legume-rhizobia symbiosis has been studied in two contrasting climatic regions, viz., extremely hot and dry desert ecosystem represented by Thar Desert, and humid ecosystem of North-Eastern sub-Himalayan region that receives the highest rainfall on the earth. An attempt has been made to understand the role of various ecological factors in the diversity and coevolution of legume-rhizobia. The study revealed successful association of native legumes with alpha-rhizobia's such as *Ensifer*, *Bradyrhizobium* and *Rhizobium* effectively fixes nitrogen and improves soil fertility in the Thar Desert ecosystem (1-4). The results also show that genetically distinct groups of Old World *Ensifer* strains have evolved in the Thar Desert which are capable of nodulating native legumes belonging to three subfamilies of Leguminosae. These novel strains of *Ensifer* possess different types of symbiosis-related genes diverged from the *E. fredii* NGR234 in most of strains and *E. arboris* type in strains of *Mimosa* and *Vachellia* (2, 4). Another significant finding of the study is the discovery of the large genome size (8.5 Mbp) and high number of genes in mobilome category in *Ensifer* sp. PC2, recovered from *Prosopis cineraria* root nodules from the Thar Desert (5). The incongruence observed in species and symbiotic gene phylogeny of desert *Ensifer* strains may be attributed to soil alkalinity and arid environmental conditions that could be major driving forces in the evolution of these strains via horizontal gene transfer (2, 4). Similar studies carried out in humid subtropical environment of North-Eastern India characterized by wide elevation gradient, acidic soil, and high precipitation, revealed that unlike the dominance of *Ensifer* species in alkaline-arid region of Western Rajasthan, the native legumes in the wet and acidic region are predominantly nodulated by novel *Bradyrhizobium* strains and fast growing strains of *Rhizobium*. Rich diversity of beta-rhizobia was also discovered from the root nodules of exotic *Mimosa pudica* of north-east (2). Genomic analysis of microsymbiont(s) from these two environments revealed that the gene richness in wet climate of north-east was significantly greater than the Western desert. In addition, several genes related to plant growth promoting traits often specific to such ecological niches as humus-rich forest floor habitats were discovered. It is concluded that the climate-driven coevolution results in the differential biogeographic patterns in microsymbiont(s).

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POSTER 9-18

Diversity of *Lupinus cosentinii* Root Nodule Endosymbiotic Bacteria in Morocco

Mustapha Missbah El Idrissi[1], Oumaima Essoufi[1], Younes Abbas[2], Zoulikha Bouchiba[3] and Abdelmoumen Hanaa[1]

[1] *Laboratoire d'Amélioration de la Productivité des Sols et Environnement, Ecole Normale Supérieure, Université Mohammed V, Rabat, Morocco*
[2] *Centre de Recherche Forestière, Haut-Commissariat aux Eaux et Forêts, Rabat, Morocco*
[3] *Laboratoire de Biotechnologie des Rhizobiums et Amélioration des Plantes, Faculté des Sciences, Université Es-Senia, Oran, Algérie*

In this work, we analyzed the phenotypic and genetic diversity of 22 isolates nodulating *Lupinus cosentinii* in the region of Rabat, Morocco.

The genetic analysis based on rep-PCR, showed a great diversity between the strains. However, the 16S rDNA sequencing analysis showed they all belong to *Microvirga* sp. with 97% similarity with *M. lupini*. The analysis of *nodC* genes sequences showed similarities with some mesorhizobia *nodC* suggesting a potential lateral gene transfer in the rhizosphere.

The housekeeping genes sequencing confirmed the divergence of the strains from known *Microvirga* species, confirming the potential belonging of the newly isolated strains to a new *Microvirga* species

The phenotypic results obtained showed the high level of diversity between the isolates and their inaptitude to use different carbohydrates as sole carbon sources, whereas some were able to assimilate starch. All the isolates were slow growing, able to grow in pH values between 6 and 9, only 20% tolerated salt concentration higher than 2%.

POSTER 9-19

Diversity of *nifH* Genes of *Rhizobium leguminosarum* bv. *trifolii* Strains Derived from 100-yrs Old Zn-Pb Waste Heap in Southern Poland

Ewa Olenska[1], Wanda Malek[2]

[1] *Department Genetics and Evolution, Institute of Biology, University of Bialystok, Bialystok, Poland*
[2] *Department of Genetics and Microbiology, Institute of Microbiology and Biotechnology, Maria Curie-Sklodowska University, Lublin, Poland*

Heavy metals, as one of the natural selection factors, may reduce a genetic polymorphism within bacteria populations (1) and in a consequence, they may shape the bacteria genetic diversity (2). The final result of a strong heavy metal selective pressure may be the determination of the bacteria specific genotypes (3). In Poland, the natural laboratories to study the microevolution processes are zinc-lead waste heaps in Olkusz region. About 70-100-yrs old Zn-Pb waste heap Boleslaw is an area where soil is highly contaminated by heavy metals, mainly zinc (Zn), lead (Pb) and cadmium (Cd) as well as highly deficient in nutrients and water (4). In spite of such disadvantageous conditions, Boleslaw waste-heap is inhabited by *Trifolium repens* of natural origin. The white clover, as a fabaceans' member, enters the symbiosis with rhizobia, which reduce unavailable to plants atmospheric nitrogen into ammonium available to them (5). This conversion is catalyzed by rhizobium nitrogenase complex composed of dinitrogenase and dinitrogenase reductase.

The aim of the present study was to determine the genetic polymorphism of white clover microsymbionts, i.e. *Rhizobium leguminosarum* bv. *trifolii* bacteria, derived from zinc-lead waste-heap Boleslaw (Silesia-Kraków Upland) and compare it to that of Bolestraszyce (Przemyskie Foothills) control population, on the basis of the dinitrogenase reductase (*nifH*) genes sequences analysis.

Among forty two strains of *R. leguminosarum* bv. *trifolii* studied for the 684-bp long *nifH* genes sequences, six genotypes, including unique ones for the contaminant and control areas as well as genotypes common to both populations, were determined. The frequency of genotypes in a waste heap rhizobium population differed substantially in comparison with that of control grassland. The genetic polymorphism level, measured as a genotype (h) and nucleotide (π) diversity index, was substantially reduced in Zn-Pb Boleslaw population (h=0.533, π=1.078) in comparison to control one (h=0.729, π=2.180). Both analyzed *R. leguminosarum* bv. *trifolii* populations were significantly differed (F_{ST}=0.159, p=0.018). The *nifH* gene sequences of studied *T. repens* symbionts exhibited the highest nucleotide identity (95–100%) with those of *R. leguminosarum* bv. *trifolii* reference strains and all these strains formed in *nifH* gene phylogram monophyletic, highly supported clade (100%).

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POSTER 9-20

Diversity of Nodule-associated Endophytic Bacteria from *Cicer arietinum* L. Grown in a Soil of Mainland Spain

Xavier Cruz-González[1], Marta Marcos-García[1], Paula García-Fraile[2], Raúl Rivas[1,3]

[1] *Department of Microbiology and Genetics, Universidad de Salamanca, Castilla y León, Spain / CIALE, USAL, Spain*
[2] *Microbiology Institute. Academy of Science of the Czech Republic. Prague, Czech Republic*
[3] *Associated I+D Unit (USAL)-CSIC (IRNASA), Spain*

After cereals, legumes are the second most important crop for human diet. One of the most important legumes is chickpea (*Cicer arietinum* L.), being essential for human diets in several countries, such as India and Mediterranean countries (1). Although chickpea is considered a restrictive host for nodulation, different studies showed that several *Mesorhizobium* species may effectively nodulate chickpea (2, 3).

Despite, the importance and the high nutritional value of this legume, there are just few studies reporting the endophytic diversity harbored in chickpea nodules (2). The aim of this work was to isolate and analyze microbial populations associated to chickpea nodules, using molecular techniques. We isolated 98 strains from effective nodules of *C. arietinum* growing in a soil from Fuentesauco (Castilla y Leon, NW Spain).

The 98 isolates were grouped within 67 different RAPD profiles, showing the high genetic diversity of the nodule-associated endophytic bacteria. Representative strains from each RAPD type were identified on the basis of 16S rRNA gene sequencing, showing that 16 of the isolates were identified within three different species of the genus *Mesorhizobium*, 5 isolates corresponded to two species of *Paenibacillus,* 32 strains belonged to four *Bacillus* species, 7 isolates were identified as *Brevibacterium halotolerans,* 2 as *Domibacillus,* 1 as *Sphaerisporangium dianthi* and the 4 remaining isolates were classified as 3 *Micromonospora* species.

Taking into account the significant variability of microorganisms co-existing in the *Cicer arietinum* nodules, the study of the possible interactions between the micro and the macro-symbionts are of great interest.

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POSTER 9-21

Diversity of Rhizobia that Nodulate Faba Bean *Vicia faba* in Morocco

Hanaa Abdelmoumen, Hanan Lamin and Mustapha Missbah-Elidrissi

Laboratoire d'Amélioration de la Productivité des sols et Environnement, Ecole Normale Supérieure, Université Mohammed V, Rabat, Morocco
missbah49@yahoo.fr

Faba bean (*Vicia faba*) is the most widely grown legume in Morocco. It has a great importance as food for humans and animals and it also has an important role as green manure. This is due to its ability to establish symbiosis with soil bacteria, nitrogen-fixing called rhizobia, which facilitates nitrogen uptake by plants.

In this study, we isolated 40 bacteria from root nodules of faba bean plants cultivated in five different soils in Morocco. The isolates were characterized phenotypically and genetically.

Phenotypic analysis involved 65 physiological and biochemical parameters and highlighted the great diversity of the isolates. The results showed the high sensitivity of the isolates to salinity and pH. However, we noticed their high tolerance to heavy metals. The isolates are capable of using a variety of sugars and amino acids, but not starch or glycine. They don't produce urease or gelatinase.

The rep-PCR using BOX A1R primer showed that the isolates could be grouped into 18 different strains. Only 25 strains do possess the *nodC* gene and were able to renodulate *Vicia faba* seedlings in axenic conditions. The 16S rDNA sequencing permitted their identification using the Blast program and NCBI database as they were close to *Rhizobium laguerreae*.

Phylogenetic analysis of the 16SrRNA and the two housekeeping genes *atpD* and *glnII* using the software MEGA5 confirmed the belonging of the strains to the genus *Rhizobium* and their similarity with the species *Rhizobium laguerreae* FB206 (NR_118274.1).

POSTER 9-22

Draft Genome Sequence and Description of *Rhizobium boleqi* sp. nov.

Aregu Amsalu Aserse[1], Tanja Woyke[2], Nikos C. Kyrpides[2], William B. Whitman[3], Kristina Lindström[1]

[1] *Department of Environmental Sciences, Viikinkaari 2a, University of Helsinki, Finland*
[2] *Joint Genome Institute, United States*
[3] *Department of Microbiology, Biological Sciences Building, University of Georgia, Athens, United States*

A draft genome sequence was generated for the prospective type strain, *Rhizobium* sp. HBR26, of a new species of common bean (*Phaseolus vulgaris*) -nodulating rhizobia isolated from bean plants in Ethiopia (1). The sequencing was a part of the DOE Joint Genome Institute (JGI) 2014 Genomic Encyclopedia project designed for soil and plant-associated newly described type strains. Based on analysis of the genome sequence of the type strain HBR26 and its annotation, we propose a new species, *Rhizobium boleqi* sp. nov. The species includes five rhizobial strains, effective nitrogen fixers in symbiosis with the host plant common bean. Its genome is arranged in 62 scaffolds and consists of 6,557,588 bp, with a 61 % G+C content. The genome contains 6,221 protein-coding and 86 RNA genes. Of the protein-coding genes, 5049 (80.05%) were assigned with putative functions. The remaining genes were annotated as hypothetical proteins. The genome of strain HBR26 is about the same size as that of *R. phaseoli* Ch24-10 (6.6Mbp) and slightly greater than those of *R. etli* CFN 42^T (6.5 Mbp) and *R. phaseoli* CIAT 652 (6.4 Mbp). The nodulation genes *nodB*, *nodC, nodS, nodI, nodJ* and *nodD* are organized in similar way as *nod* genes found in the genome of other common bean-nodulating rhizobial strains such as CFN 42^T, Ch24-10, CIAT 652, *R. leguminosarum* sv. *phaseoli* CCGM1 and *R. etli* sv. *phaseoli* IE4803. The *nod* genes and most nitrogen-fixing genes found in the genome of HBR26 shared high identity with corresponding genes of CFN 42^T, CIA 652, CCGM1 or Ch24-10 (96-100% identity), suggesting that symbiotic genes might be shared between these rhizobia through horizontal gene transfer. On the other hand, genome Average Nucleotide Identity (gANI) calculation based on protein coding genes revealed that *R. etli* CFN 42^T with 90.2% gANI is the closest sequenced strain followed by *R. etli* sv. *mimosae* Mim1 (89.6%). In our previous study based on multiple housekeeping protein coding gene analyses, *R. etli* was also the closest rhizobial species to the novel species (1). Nevertheless, the gANI value between HBR26 and CFN 42^T is by far lower than the cut off value of ANI (>=96) between strains in the same species (2), confirming that HBR26 belongs to novel species. Data on the genome assembly and annotation is available in the JGI portal (http://genome.jgi.doe.gov/). The presentation will also include structural genomic analysis comparing the HBR26 genome with reference genomes, visualization of the genomic features and Venn diagram showing orthologous clusters of protein coding genes.

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POSTER 9-23

Genetic Diversity and Structure of Native Rhizobia Associated with *Medicago spp.* Plants

Andrei Stefan[1], Jannick Van Cauwenberghe[2,3], Craita M. Rosu[1], Catalina Stedel[1,4], Nikolaos E. Labrou[5], Emmanouil Flemetakis[5], Rodica C. Efrose[1]

[1] *Department of Experimental and Applied Biology, NIRDBS-Institute of Biological Research Iasi, Iasi, Romania*
[2] *Programa de Genómica Evolutiva, Centro de Ciencias Genómicas, UNAM, Cuernavaca, Morelos, México*
[3] *Plant Conservation and Population Biology, Biology Department, KU Leuven, Leuven, Belgium*
[4] *Department of Biochemistry & Biotechnology, University of Tessaly, Larissa, Greece*
[5] *Department of Biotechnology, School of Food, Biotechnology & Development, Agricultural University of Athens, Athens, Greece*

Alfalfa is a member of the *Fabaceae* family capable of establishing nitrogen-fixing symbioses with a broad range of rhizobial species. In Romania, alfalfa occupies the largest cultivated surface of any legumes and is grown mainly as fodder. In this study we assessed 67 rhizobial isolates associated with three cultivated or wildly grown *Medicago* species (*M. sativa*, *M. falcata* and *M. lupulina*) and originating from three distinct geographical locations in Eastern Romania. A multilocus sequence analysis (MLSA) performed on three concatenated housekeeping genes, *atpD*, *glnII* and *recA* delineated the 67 rhizobial isolates into five confident branches which confirmed the *16S rRNA* clustering. Phylogenetic analysis revealed a high diversity of the native isolates which were affiliated to *Sinorhizobium meliloti* (73.1%), *Rhizobium leguminosarum* (13.4%), *Sinorhizobium medicae* (5.9%), *Mesorhizobium sp.* (5.9%) and *Agrobacterium sp.* (1.5%). Additional accessory genes (*nodA* and *nifH*) were used in order to assess the establishment of efficient symbiosis. A hierarchical analysis of molecular variance (AMOVA) revealed that the genetic diversity of the rhizobial populations was influenced by the presence of certain host-plants and geographical location. Rhizobial population structure analysis assigned the rhizobial isolates into three distinct gene pools with different degrees of admixture.

POSTER 9-24

Lupin-nodulating Rhizobia Isolated from *Lupinus* spp. Native to the Andes and California Carry Phylogenetically Distinct Symbiotic Loci

Joanna Banasiewicz, Klaudia Komendacka, Joanna Śliwka, Tomasz Stępkowski

Faculty of Agriculture and Biology, Warsaw University of Life Sciences, Warsaw, Poland

Legume species belonging to the genus *Lupinus* form the nitrogen-fixing symbiosis with a relatively broad range of rhizobium genera and species. The most common are the isolates of the genus *Bradyrhizobium*, which seems to be the dominant group among rhizobium symbionts nodulating lupins (1,2). Much less is known about fast-growing rhizobium symbionts of this legume genus. Given that beta-rhizobia have not been reported among lupin rhizobia, it seems that they belong exclusively to the alpha-Proteobacteria, representing the genera; *Mesorhizobium*, *Microvirga*, and possibly, *Rhizobium*, *Phyllobacterium*, and *Ochrobactrum* (3,4).

In this study, we report the characterization of rhizobium strains isolated from lupin species that are native, respectively, to the Andes and California. The rhizobium isolates were characterized using multilocus sequence analysis (MLSA) approach, based on the generation of partial sequences of several core genes (*recA*, *glnII*, *gyrB*, *rpoB* and 16S rRNA), as well as on amplification of symbiotic *nodA*, *nodZ*, *nifD* and *nifH* loci. Preliminary data indicate that although 16S rRNA sequences of the isolates from the Andean lupin show 99% identity to *Microvirga zambiensis* strain WSM3693, the symbiotic *nodA*, and *nodZ* gene sequences are clearly distinct from this and other rhizobium genera sequences. On the other hand, core gene sequences of the isolates from native to California *Lupinus arboreus* show phylogenetic affinity to *Mesorhizobium ciceri*, having the nodulation *nodA* gene sequences that are also quite dissimilar with respect to those of other rhizobia. This study indicates that symbiotic loci of lupin rhizobia in the two primary centers of *Lupinus* diversification are highly diverse, which may suggest a linkage between the differentiation of rhizobial symbionts and explosive radiation of this legume genus (5).

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POSTER 9-25

Phenotypic Characterization of Abiotic Stress Tolerant *Ensifer* Species Nodulating *Phaseolus filiformis* in Arid Soils of Northern Mexico

Guadalupe Rocha[1], Ricardo Carreño[1], José-Luis Contreras[2], María-del-Carmen Villegas[3], Clémence Chaintreuil[4], Bernard Dreyfus[4], Antoine Le Queré[4], José-Antonio Munive[1]

[1] *Centro de Investigaciones en Ciencias Microbiológicas, Instituto de Ciencias, Benemérita Universidad Autónoma de Puebla, México*
[2] *Facultad de Arquitectura, Benemérita Universidad Autónoma de Puebla, México*
[3] *Laboratorio de Ecología Microbiana, Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, México*
[4] *Laboratoire des Symbioses Tropicales et Méditerranéennes, IRD/ CIRAD/ UM2/ Supagro-UR040, Montpellier, France*

In northern Mexico, the dry climate, salinity and high temperatures, limit areas that can be cultivated. The aim of the present work was to characterize salt tolerant rhizobia nodulating *Phaseolus filiformis* in saline soils of Baja California desert. *P. filiformis* is a wild bean species native to the southwestern United States and northwestern Mexico. Rhizobial species nodulating this legume were identified as belonging to genus *Ensifer* by 16SrDNA sequence analysis. Phenotypic characterization revealed that *Ensifer* populations associated with *P. filiformis* in Mexico are salt tolerant bacteria, able to grow on alkaline conditions and high temperatures. These strains can develop the symbiosis with 5 cultivated bean varieties tested. The phylogenetic analysis of *nod*C sequences showed that Mexican isolates carry symbiotic genes divergent from those previously characterized among bean symbionts. These were closely related to *nod*C sequences of *E. americanum* strains isolated from *Acacia macracantha* nodules in Peru and more distantly to *nod*C sequences of *E. meliloti* strains belonging to biovar mediterranense, and *E. americanum* symbionts of *P. vulgaris*. The high similarity between symbiotic genes sequences found in *P. filiformis* rhizobia suggest that these genes have the same origin and diverged recently. These strains are capable to utilize a wide range of carbohydrates and organic acids as sole carbon sources for growth, but they are unable to grow on glycogen. This study improves the knowledge on diversity, geographic distribution and evolution of bean-nodulating rhizobia in Mexico, and further enlarges the spectrum of microsymbiont with which *Phaseolus* species can interact with.

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POSTER 9-26

Phylogenetic Diversity of *Mesorhizobium* Strains Nodulating *L. corniculatus* and its Ability to Re-infect the Host

Marta Marcos-García[1], Esther Menéndez[1], Xavier Cruz-González[1], Encarna Velázquez[1, 2], Pedro F. Mateos[1, 2], Raúl Rivas[1, 2]

[1] *Department of Microbiology and Genetics, University of Salamanca (USAL), Spain / CIALE, USAL, Spain*
[2] *Associated I+D Unit (USAL)-CSIC (IRNASA), Spain*

Lotus corniculatus is a perennial legume used for pasture and silage production in many temperate countries (1). It establishes a nitrogen-fixing symbiosis with rhizobia than have been scarcely studied, despite of its value as fodder for animals (2). Three species of *Mesorhizobium* genus are endosymbionts of this legume: *M. loti*, *M. erdmanii* and *M. jarvisii*, which were initially considered to be the same species (3).

In the present study we analysed the genetic diversity of strains nodulating *L. corniculatus*, isolated from several effective nodules in plants from this legume, collected at flowering stage in Carbajosa de la Sagrada, a Mediterranean region from Salamanca (NW Spain). The phylogenetic analysis of the 16S rRNA gene, of strains displaying different RAPD patterns, showed that they belong to divergent phylogenetic groups within the genus *Mesorhizobium*, which are widely distributed worldwide (4). The analysis of the *recA* and *atpD* genes showed that our strains belong to several clusters, one of them very closely related to *M. jarvisii* and the remaining ones phylogenetically divergent from the currently described *Mesorhizobium* species (4). These results revealed that *L. corniculatus* plants collected in NW Spain harbour strains with a great phylogenetic diversity, comparable to that found in other studies performed at different continents and countries (5).

Furthermore, we performed re-infection assays in *L. corniculatus* plants. As expected, all strains isolated were able to re-induce effective nodules in this host, although their nodulation efficacy was diverse, as showed by the obtained data from nodule number. In nodule sections, we observed a good organization with more or less occupancy of the legume invaded cells, depending on the strain. We observed differences in shoot length among treatments, as well as with respect to the N-free uninoculated treatment plants. These results showed the efficiency of the symbiosis *L. corniculatus* - *Mesorhizobium* in NW Spain soils, allowing us to select strains for future microcosms and field experiments.

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POSTER 9-27

Phylogeny and Molecular Identification of *Ensifer* species Nodulating *Phaseolus filiformis* in Northern Mexico on the Basis of Multilocus Sequence Analysis

Guadalupe Rocha[1], Ricardo Carreño[1], Rocío Bustillos[1], Jesús Muñoz[1], Clémence Chaintreuil[2], Bernard Dreyfus[2], Antoine Le Queré[2], José-Antonio Munive[1]

[1] *Centro de Investigaciones en Ciencias Microbiológicas, Instituto de Ciencias, Benemérita Universidad Autónoma de Puebla, México*
[2] *Laboratoire des Symbioses Tropicales et Méditerranéennes, IRD/ CIRAD/ UM2/ Supagro-UR040, Montpellier, France*

The genus *Phaseolus* comprises around 50 species, all indigenous to the Americas. Among these, common bean (*P. vulgaris* L.) represents the most important source of proteins for low-income populations in Latin America and Africa. A remarkable attribute of common bean is its ability to nodulate with diverse rhizobia. Most reported phylogenies of rhizobia nodulating *P. vulgaris* have placed them in the genus *Rhizobium* and it has been reported that strains outside this genus formed ineffective symbiosis with beans. However, later studies have shown that strains belonging to the genera *Ensifer* (*Sinorhizobium*) (*E. fredii*, *E. meliloti* and more recently *E. americanum*) could also develop an effective symbiosis with this crop. A number of rhizobia strains were isolated from *Phaseolus filiformis* (a wild bean native to the southwestern United States and northern Mexico) growing in alkaline soils in Baja California (Mexico). By analyzing the 16SrRNA sequences, we found that these rhizobacteria belong to the genus *Ensifer*. In order to clarify the taxonomic position of these Mexican isolates, considering that the use of additional loci present in single copies on genomes with greater sequence divergence and evenly distributed in the genome is an alternative approach that overcomes the drawbacks of 16SrRNA based analyses, eight gene fragments (*atp*D, *dna*K, *rpo*B, *gln*A, *gyr*B, *thr*C, *rec*A and *16SrRNA*) were chosen to perform a Multilocus Sequence Analysis (MLSA). The phylogenetic relationships obtained indicate that there are possibly several species among the isolated strains, showing clearly that *Ensifer* strains isolated from alkaline sandy soils in Baja California, are divergent from *E. americanum* species (dominant *P. vulgaris* symbiont isolated from alkaline soils in Mexico) but further support a better adaptation of *Ensifer* species toward basic pH.

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POSTER 9-28

Population Genetics of *Rhizobium leguminosarum* Based on 192 *de novo* Assembled Genomes

Asger Bachmann[1], Sara Moeskjær[1], Bjarni Vilhjalmsson[2], Luc Janns[1], Mikkel H. Schierup[2], J. Peter W. Young[3] and Stig U. Andersen[1]

[1] *Department for Molecular Biology and Genetics, Aarhus Universitet, Denmark*
[2] *Bioinformatics Research Center, Aarhus University, Denmark*
[3] *University of York, York, United Kingdom*

As part of the NCHAIN project (http://mbg.au.dk/nchain), which aims to optimize nitrogen fixation through genomic prediction of the effects of interspecies interations, we have isolated 248 rhizobium strains from white clover root nodules. 192 representative strains were sequenced using 2x250 bp Illumina reads and assembled *de novo* into 80-200 contigs per strain. Assigning chromosomal or plasmid origin to these contigs has allowed us to evaluate genetic diversity and linkage taking into account the genomic context. We have found evidence of extensive recombination within genes and have used population genetics methods to investigate the nature of the selective pressures imposed on rhizobium genomes by the plant host via the rhizobium symbiosis genes. Furthermore, we have assessed the importance of auxiliary genes in adaptation to geographical location and to specific symbiotic genotypes.

POSTER 9-29

Proficiency to Transfer the Symbiosis Island is a Bistable pheNotype in *Mesorhizobium loti* Strain R7A Populations

Clive W. Ronson[1], John T. Sullivan[1], Liam Harold[1], Dinah D. Tambalo[2], Christopher K. Yost[2], Michael F. Hynes[3], Joshua P. Ramsay[4]

[1] *Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand*
[2] *Biology Department, University of Regina, Regina, Canada*
[3] *Department of Biological Sciences, University of Calgary, Calgary, Canada*
[4] *School of Biomedical Sciences, Curtin University, Perth, Australia*

Symbiosis islands are integrative and conjugative mobile genetic elements (ICEs) that convert nonsymbiotic rhizobia into nitrogen-fixing symbionts of leguminous plants. Excision and horizontal transfer of the *Mesorhizobium loti* symbiosis island ICE*Ml*Sym^{R7A} is indirectly activated by quorum sensing through TraR-dependent activation of the excisionase gene *rdfS* in the presence of the N-acyl-homoserine lactone 3-oxo-C6-HSL (1). The presence of additional copies of TraR stimulates 3-oxo-C6-HSL production and leads to ICE excision in 100% of cells, with corresponding high transfer frequencies. However, in wild-type cultures, 3-oxo-C6-HSL production only occurs at a very low level (undetectable using the indicator strain *Chromobacterium violaceum* CV056), the ICE is excised in less than 1% of cells, and transfer frequency is very low (1). This is due to repression by QseM, a widely conserved ICE-encoded protein that is an antiactivator of TraR (2) and the downstream excision activator FseA (3). *qseM* expression is repressed by a DNA-binding protein QseC, which also activates *qseC* expression from a leaderless transcript. QseC differentially binds two adjacent operator sites, the lower affinity of which overlaps the -35 regions of the divergent *qseC-qseM* promoters (2). On the basis of these results, we proposed that QseC and QseM comprise a bimodal switch that restricts quorum sensing and ICE*Ml*Sym^{R7A} transfer to a small proportion of cells in the population (2,3,4). In this work, we isolated a derivative of *M. loti* R7A, strain R7A*, that produced 3-oxo-C6-HSL at a high level and transferred ICE*Ml*Sym^{R7A} at a high rate. Sequencing of R7A* failed to reveal any mutations and transfer experiments showed that the R7A* phenotypes were not conferred by either the island or the core chromosome. Further work showed that about one in 500 colonies derived from plating a wild-type R7A culture exhibited R7A*-like phenotypes. These derivatives when subcultured continued to express these phenotypes in a stable manner. RNA-Seq experiments showed differential expression of *qseM* and other genes between R7A and R7A*. We propose that the transfer competence of a *M. loti* R7A population is a bistable state with the minority transfer-proficient state, represented by R7A*, due to prolonged repression of *qseM* as a result of production of QseC above a threshold level followed by positive autoregulation. This system may facilitate a bet-hedging strategy by ICE*Ml*Sym^{R7A}, in which only a small proportion of cells in the population can respond to 3-oxo-C6-HSL and act as donors in the physiologically expensive and risky process of transfer.

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POSTER 9-30

Rhizobium altiplani, New Species Isolated from Root Nodules of *Mimosa pudica* in Brazil

Alexandre C. Baraúna[1], Luc F. M. Rouws[2], Jean L. Simões-Araujo[2], Fábio B. dos Reis Junior[3], Pietro P.M. Iannetta[4], Marta M. Maluk[4], Silvia R. Goi[1], Veronica M. Reis[2], Euan K. James[4], Jerri E. Zilli[2*].

[1] *Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brazil*
[2] *Embrapa Agrobiologia, Seropédica, Rio de Janeiro, Brazil*
[3] *Embrapa Cerrados, Planaltina, Brazil*
[4] *The James Hutton Institute, Invergowrie, Dundee, UK.*

Root nodule bacteria were isolated from nodules on *Mimosa pudica* L. growing in neutral-alkaline soils from the Distrito Federal in Central Brazil. The 16S rRNA gene sequence analysis of ten strains placed them into the genus *Rhizobium* with the closest neighbouring species (each with 99% similarity) being *R. grahamii, R. cauense, R. mesoamericanum* and *R. tibeticum*. This high similarity, however, was not confirmed by multi locus sequence analysis (MLSA) using three housekeeping genes (*recA, glnII* and *rpoB*), which revealed *R. mesoamericanum* strain CCGE501^T to be the closest type strain (92% sequence similarity or less). Chemotaxonomic data, including fatty acid profiles (with majority being C $_{19:0}$ cyclo w8c and Summed Feature 8 ($C_{18:1}$ w6c), DNA G+C content (57.58% mol), and carbon compound utilization patterns supported the placement of the novel strains in the genus *Rhizobium*. Results of Average Nucleotide Identity (ANI) differentiated the novel strains from the closest *Rhizobium* species, *R. mesoamericanum*, *R. grahamii*, and *R. tibeticum* with 89.04, 88.08 and 87.75 % similarity, respectively. A symbiotic gene essential for symbiotic nodulation (*nodC*) and nitrogen fixation (*nifH*) were most similar (99-100 %) to those of *R. mesoamericanum*, another *Mimosa*-nodulating species. Based on the current data, ten strains represent a novel species for which the name *Rhizobium altiplani* sp. nov. (BR10423^T = HAMBI 3664^T) is proposed.

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POSTER 9-31

SOILMAN - Mapping, Management and Resilience of Ecosystem Services for Food Security and Response to Climate Change in Ethiopia

Kristina Lindström[1], Aregu Aserse[1], Markku Yli-Halla[2], Mila Sell[1,3], Beimnet Hailemariam[1,4], Asefa Fassil[4], Daniel Markos[5], Gennet Getachew[5], Galfato Gabiso[6], Zebene Asfaw[6]

[1] *Department of Environmental Sciences and Department of Food and Environmental Sciences, Finland*
[2] *University of Helsinki, Finland*
[3] *Natural Resources Institute Finland (Luke), Finland*
[4] *Department of Cellular, Microbial and Molecular Biology, Addis Ababa University, Ethiopia*
[5] *Southern Agricultural Research Institute, Hawassa, Ethiopia*
[6] *Hawassa University, Hawassa, Ethiopia*

Ethiopia is endowed with a variety of agro-ecological zones that differ in terms of rainfall, temperature, altitude, soil types and vegetation. These have resulted in rich species diversity including many cultivated and wild plant, and also great variation in the agricultural activities carried out in different agro-ecological zones. The country is considered as the centre of origin and diversity of many cultivated crops; and hence recognized as one of the Vavilovian crop diversity centres. Most of these crops have their origin in the country and maintain high levels of diversity with the presence of several local landraces restricted to certain agro-ecologies. The country is also regarded as centre of diversity for several legumes, and their associated nitrogen-fixing rhizobium bacteria. SOILMAN aims at making use of this diversity for improving food security, helping restore degraded environments and mitigating climate change with the help of biological nitrogen fixation (BNF) in the symbiosis between rhizobium bacteria and legumes and by involving farmers in the process.

SOILMAN is divided into four subprojects: (i) Genomics and taxonomy of rhizobia isolated from common bean (*Phaseolus vulgaris)*, *Crotalaria* spp., *Indigofera* spp. and *Erythrina brucei* growing in Ethiopia. These bacteria formed unique phylogenetic groups that were distinct from recognized rhizobial species based on MLSA (1,2,3) and genome sequencing is now used to characterize new species. (ii) Collection and characterization of rhizobia from diverse Ethiopian soils differing in clay content, acidity and salinity, by trapping with *Cicer arietinum, Lens culinaris, Vicia faba, Lathyrus sativus* and *Cajanus cajan* for biogeographic studies and future use in agriculture. (iii) Field experiments on two Ethiopian sites (loam soils with pH 6.4 and 8.0) with common bean and soybean (*Glycine max*) and strains from the Ethiopian collection that have performed well in greenhouse conditions. (iv) Work with farmers and other stakeholders in an Innovation Platform process. Selected farmers and extension workers from two villages meet regularly with our facilitator to learn about legume cultivation and inoculation and to share experience on their use. In the first year farmers were offered seed and inoculant for common bean and soybean, a novelty in the area. Only four farmers chose soybean then, but in the second year almost everybody wanted

it. A knowledge workshop on the nutritional value of soybean followed by a cooking workshop still boosted the interest in soybean. Some farmers set up small experiments to investigate nodulation and the interest was spread to neighbours. To sustain the activities in the future, we involved a local cooperative organisation. Locally produced, high-quality inoculant must be secured and access to markets ensured.

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POSTER 9-32

Strategies for Increased Ammonium Production in Free-living or Plant Associated Nitrogen Fixing Bacteria

Florence Mus[1], Alex Tseng[1], Philip S. Poole[2], Ray Dixon[3] and J. W. Peters[1]

[1] *Department of Chemistry and Biochemistry, Montana State University, Bozeman, United States*
[2] *Department of Plant Sciences, University of Oxford, Oxford, United Kingdom*
[3] *John Innes Centre, Norwich Research Park, Norwich, United Kingdom*
florence.mus@.montana.edu

Overcoming the inhibitory effects of excess environmental ammonium on nitrogenase synthesis or activity, or preventing ammonium assimilation, have both been considered as strategies to increase the amount of fixed nitrogen transferred from bacteria to plant partners in associative or symbiotic plant-diazotroph relationships.

GlnE adenylyltransferase catalyzes reversible adenylylation of glutamine synthetase (GS), thereby affecting the post-translational regulation of nitrogen assimilation critical for appropriate coordination of carbon and nitrogen assimilation. Since GS is of such crucial importance for the cell, attempts to obtain *Azotobacter vinelandii* deletion mutants in the gene encoding GS (*glnA*) have been unsuccessful. We have generated a *glnE* deletion strain presumably unable to adenylylate GS, thus preventing the post-translational regulation of GS. The resultant strain containing constitutively active GS is unable to grow well on ammonium-containing medium as previously observed in other organisms and can be cultured only at low ammonia concentrations. This phenotype is caused by the lack of down regulation of GS activity resulting in depletion of the 2-oxoglutarate pools and cessation of metabolism. Interestingly, the mutant can grow diazotropically with rates comparable to wild-type. This observation suggests that the control of nitrogen fixation specific gene expression at the transcriptional level in response to 2-oxoglutarate via NifA is sufficiently tight to alone regulate ammonia production at appropriate levels for optimal carbon and nitrogen balance.

In part the motivation for revisiting the regulation of nitrogen assimilation and nitrogen fixation is in the vain of biotechnological solutions and the engineering of ammonia excreting diazotrophs to associate with various crop plants. The *glnE* deletion mutant can be used in this project as a tool to examine the threshold of ammonia required and/or tolerated under a specific set of growth conditions, and to identify mutant strains with higher titers of ammonia tolerance.

This work is part of the SynSym project which aims to engineer interactions between nitrogen-fixing bacteria and plants. This work was supported by the National Science Foundation [grant number NSF-1331098]; and the Biotechnology and Biological Sciences Research Council [grant numbers BB/L011484/1, BB/L011476/1].

POSTER 9-33

Biological Nitrogen Fixation Book Volume I and II

Frans J. de Bruijn

INRA/CNRS Laboratory of Plant-Microbe Interactions (LIPM), Chemin de Borde Rouge, Auzeville B.P. 52627, 31326 Castanet-Tolosan Cedex, France

Nitrogen is arguably the most important nutrient required by plants, being an essential component of all aminoacids and nucleic acids. However, the availability of nitrogen is limited in many soils and although the earth's atmosphere consists of 78.1% nitrogen gas (N2) plants are unable to use this form of nitrogen. To compensate, modern agriculture has been highly reliant on industrial nitrogen fertilizers to achieve maximum crop productivity. However, a great deal of fossil fuel is required for the production and delivery of nitrogen fertilizer. Indeed industrial nitrogen fixation alone accounts for 50% of fossil fuel used in agriculture. Moreover, N-fertiliser production and use present environmental problems, such as greenhouse gas production (CO2 and NOx) and waterway eutrophication. Thus there is a strong need to reduce our reliance on chemical chemical nitrogen fertilizers and instead optimize alternative nitrogen inputs. Biological nitrogen fixation is one alternative to nitrogen fertilizer. It is this process and its major players which will be discussed in the "Biological Nitrogen Fixation" Book (de Bruijn, 2015). The best known and most extensively studied example of biological nitrogen fixation is the symbiotic interaction between nitrogen fixing "rhizobia" and legume plants. It is this symbiotic interaction which will be highlighted in the Book. It will also include chapters on the taxonomy, and evolution of diazotrophs, as well as their physiology and metabolism. It will feature chapters on the genomics, transcriptomics and proteomics of diazotrophs and (host) plants and present chapters on inoculum production and field studies. While legumes are important as major food and feed crops and are the second group of such crops grown worldwide, the first group (cereals such as wheat, mays and rice) does not have this symbiotic nitrogen fixing interaction with rhizobia. It has thus been a "holy grail" to transfer the ability to fix nitrogen to the cereals and different timely approaches towards this goal are also discussed in the Book. The book brings together both review and original research articles on key topics in nitrogen fixation. This text covers the full breath of current aspects of biological nitrogen fixation research, synthesizing it to point the way forward.

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