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MICROBIAL NITROGEN FIXATION IN THE CHANGING OCEAN

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Microbial nitrogen (N_2) fixation is the main source of new nitrogen to the ocean and plays a key role in fuelling the biological carbon pump that drives CO_2 sequestration. Major challenges in marine N_2 fixation research are to identify the main organisms responsible for this process in the ocean, to study their link to other organisms and to determine the environmental factors regulating their activity. To address these challenges, we use single cell techniques, such as in situ hybridization and nanoscale secondary ion mass spectrometry (nanoSIMS). This approach recently revealed that a terrestrial-type N_2-fixing symbiosis between seagrass and a marine bacterium plays a key role for coastal seagrass ecosystems. Furthermore, the development of a new nanoSIMS approach combined with elemental analysis of individual cells allowed us for the first time to determine single-cell iron uptake rates simultaneously to CO_2 and N_2 fixation rates in the environment. Using this novel approach, we discovered that Fe-addition induces very different physiological responses in individual members of complex N_2-fixing communities in Fe-depleted surface waters. I will discuss how these results might help us better predict future changes in the global carbon and nitrogen cycles resulting from human activities.

PL 1

INSIGHTS INTO THE MECHANISM OF N_2 REDUCTION CATALYZED BY NITROGENASE

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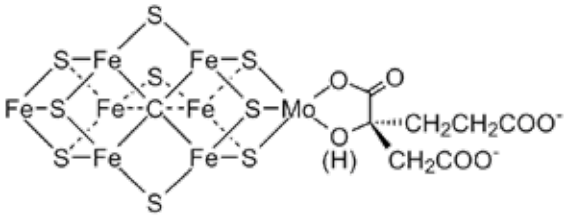
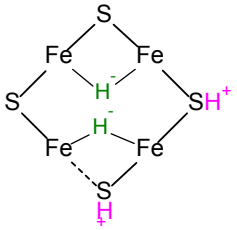
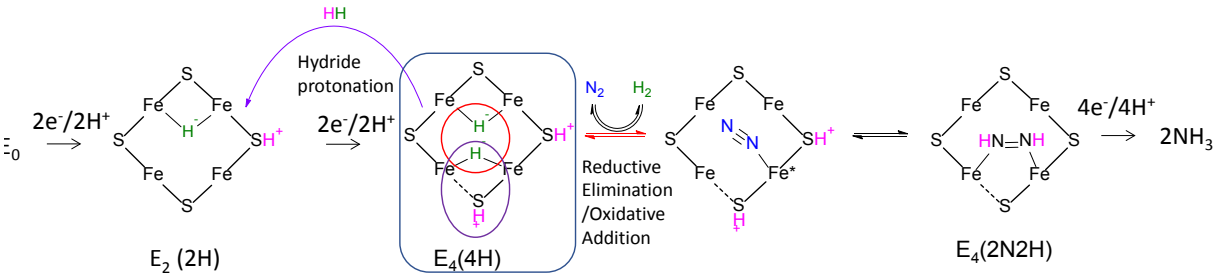
In this presentation, recent insights into the mechanism of N_2 reduction catalyzed by nitrogenase will be presented. A combination of approaches, including genetics, protein chemistry, spectroscopy, and theory are revealing several aspects of the nitrogenase mechanism at the molecular level. The accumulation of electrons and protons at the active site for the three nitrogenase isozymes (Mo, V, and Fe) occurs through formation of active site cofactor Fe-hydrides and protonation of sulfides. This activated state (E_4) can undergo relaxation back to more oxidized states by formation of H_2 or it can bind N_2 with the obligate formation of H_2. Subsequent reduction of this N_2-bound state results in the release of 2 NH_3 and recovery of the resting state of the active site. Recent studies[1,2] are providing insights into these competing reactions.







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PL 2

ENGINEERING OF NITROGENASE IN EUKARYOTES

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Nitrogen fertilization is often used to increase crop productivity, causing groundwater pollution and greenhouse gas emission. One strategy to increase biological nitrogen fixation in cereals that have little associative relationship with nitrogen-fixing organisms is the transfer and incorporation of prokaryotic nitrogenase genes into the plant genome.

Nitrogenase harbors three distinct metal prosthetic groups that are required for electron transport and N_2 reduction. The simplest is a [4Fe-4S] cluster located on the Fe protein (NifH) nitrogenase component. The MoFe protein component (NifDK) carries an [8Fe-7S] group called P-cluster and a [7Fe-9S-C-Mo-*R*-homocitrate] group called FeMo-co. Formation of active nitrogenase requires the participation of several accessory proteins. In addition, nitrogenase clusters are very sensitive to O_2, which makes it challenging to engineer nitrogenase in plant cells. To overcome the oxygenic hostile environment of the eukaryotic cell, we target the expressed nitrogenase proteins to mitochondria, where respiration could protect its metalloclusters by consuming O_2.

The central proteins for FeMo-co biosynthesis are NifB, NifEN and NifH. NifB is a radical S-adenosylmethionine (SAM) enzyme that catalyzes the synthesis of the [8Fe-9S-C] NifB-co using [4Fe-4S] clusters donated by NifU, SAM, and a sulfur source. NifEN functions as a scaffold protein that incorporates Mo and homocitrate into NifB-co in a series of reactions that also require NifH, creating FeMo-co that is then donated to apo-NifDK for activation.

We have identified variants of NifB, NifH, and NifEN that exhibit superior properties in terms of solubility, stability, O_2 resistance and functionality in yeast and plant mitochondria. We demonstrate that these proteins accumulate metalloclusters *in vivo* and are functional *in vitro* when isolated from mitochondria of yeast, tobacco, and transgenic rice, representing the first important steps towards engineering a functional nitrogenase in a major cereal crop.

PL 3

STRUCTURE AND FUNCTION OF SYMBIOTIC CELL-SURFACE RECEPTORS

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Microbial derived carbohydrates serve as signal molecules, which in legumes are perceived by cell-surface receptors. Receptor-mediated perception of chitin polymers, lipochitooligosaccharides, and exopolysaccharides initiates different signalling responses that enable the plant to distinguish beneficial from pathogenic microbes. Using structural biology, biochemistry and functional experiments our research aims to understand how these receptors work at the molecular level. I will present our recent findings on how symbiotic signals are perceived to activate the downstream pathways and highlight some of the future challenges.

PL 4

ALL PARENTS, ALL DIFFERENT: QUESTIONING ON THE MANY VARIATIONS OF *SINORHIZOBIUM MELILOTI* STRAINS

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Many molecular signals are exchanged between rhizobia and the host legume plant; some of them are crucial for the symbiosis to take place, while others are modifiers of the interaction. These latter can have great importance in competition with the soil microbiota and in genotype-specific perception of host plant[1].

Data on strain-specific and host genotype-specific interaction between rhizobia and legumes are shedding light on the genetic determinants of such modifiers, pointing to the relevance of transcriptional variation and the dispensable genome pool[2]. More recent findings are also showing the presence of rhizobial strain-specific interaction with fungi at the level of the rhizosphere microbiota. Finally, strain-by-strain epigenomic variation is emerging as a novel factor of rhizobial genomic variability, which could influence phenotypic plasticity, host plant symbiotic interaction and strain differentiation.

Here, we will discuss results emerging from such studies from the perspective of evolutionary interpreting the existence of the high genomic variation in the symbiotic rhizobium *Sinorhizobium meliloti* and its exploitation in the tailored design of synthetic communities[3].

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PL 5

EVOLUTION OF LEGUME SYMBIONTS: GENETIC BASES AND SELECTIVE FORCES

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To get insight into the evolutionary mechanisms that have facilitated the diversification of nitrogen-fixing legume symbionts, we mimicked the emergence of a new rhizobial genus under laboratory condition. For this, we transferred the symbiotic plasmid of the *Mimosa pudica* symbiont *Cupriavidus taiwanensis* into *Ralstonia solanacearum* and evolved this chimera using serial inoculation-nodulation cycles on *M. pudica*. Such cycles have rapidly selected microbial variants with improved nodulation and infection capacities. Using a population-based analysis approach, we described the dynamics of this adaptive process and showed that bacterial adaptation was predominantly driven by a plant-mediated selection process of the most competitive symbionts for nodulation. This effect emerges from the presence of a selective bottleneck at host entry, the strength of which strongly influences the evolutionary trajectory of symbiont populations and probably plays a major role in the evolution of new rhizobia. Interestingly, the most adaptive mutations improved both nodulation competitiveness and nodule cell infection, showing recurrent genetic coupling between these two traits. After 60 cycles of evolution, mutualism was not achieved, in part because intracellular persistence, *i.e.* the capacity of bacteroids to persist within nodule cells, has not been completely gained. We hypothesize that important genes enabling persistence and nitrogen fixation in the natural symbiont *C. taiwanensis* might be absent in *R. solanacearum*, thus preventing the evolution of this trait. In parallel, we analyzed the plant transcriptomic responses to natural and experimentally evolved *Mimosa* symbionts. Strikingly, we identified hundreds of *Mimosa*-specific genes encoding small proteins whose expression is associated with the intracellular release and persistence of bacteroids. Whether these small proteins actually target bacteroids and are involved in the lack of persistence of our *Ralstonia* evolved clones remains to be elucidated. These small proteins are different from the Nodule Cysteine-Rich peptides identified in *Medicago*, which supports the hypothesis of a convergent evolution of symbiosome release in two legume lineages, the Mimosoideae and the Papilionoideae.

PL 6

THE MOLECULAR MECHANISMS ENABLING INTRACELLULAR RHIZOBIAL INFECTIONS IN LEGUMES

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A key feature of the evolutionary maintained ability to efficiently fix atmospheric nitrogen is the intracellular colonization of rhizobia in nodules. The cellular entry is initiated by an invagination of the plasma membrane demarcating the onset of infection thread (IT) development, the stabilization of the IT and its transcellular passage as well as the release of rhizobia into nodule cells. Over the last years we have gained a mechanistic understanding of these processes [1-4] and found that it is only a small set of cells that maintains a state of infection competence [5]. In my talk, I will summarize and merge these finding to draw a comprehensive picture of intracellular infections in legumes and provide a perspective for engineering these features into non-legumes.

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PL 7

SINGLE-CELL APPROACHES FOR DISSECTING SYMBIOTIC SIGNALLING

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In the legume-rhizobium symbiosis, relatively few root cells are successfully infected by rhizobia. This makes it difficult to accurately assess the transcriptional response of infected cells by whole-root transcriptional profiling. Instead, we have used single-cell RNA-sequencing to identify specific populations of root cells responding to rhizobium infection, including infected root hair and cortical cells[1]. Genes specifically expressed in these cells are likely to be involved in symbiotic signalling, and the approach has proven useful for identification of novel nodulation genes. We have also investigated nodulation mutants, semi-compatible interactions and shoot responses at the single-cell level. The initial results of these experiments will be discussed.

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PL 8

REGULATION OF NUCLEAR CALCIUM SIGNALLING IN ENDOSYMBIOSES

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Nutrient acquisition is fundamental to life. Plants evolved strategies to overcome soil phosphate limitation and access to atmospheric dinitrogen by developing beneficial association with root arbuscular mycorrhiza and bacteria endosymbionts, respectively. One of the hallmarks of root endosymbioses is the activation of nuclear calcium oscillations which are essential to reprogram the cells and set off endosymbioses. However, calcium is a ubiquitous secondary messenger, and our recent findings demonstrate that nuclear calcium releases not only occur to specify endosymbioses program but also is associated with root apical meristem development using the same nuclear ion channels [1,2]. How similar ion channels are regulated to generate different output is unknown. Here, recent advance in understanding how nuclear calcium oscillation is generated in endosymbioses will be presented [3].

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PL 9

RHIZOBIA ON THE MOVE: SIGNALLING AND CELLULAR RESPONSES GUIDING TRANSCELLULAR HOST INFECTION

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Legumes engage in mutualistic associations with rhizobia to establish sustainable nitrogen-fixing root symbiosis that forms specialized nitrogen-fixing nodules. Tightly controlled entry of rhizobia microsymbionts into roots is a crucial early step in this interaction and occurs in most legumes via polar tip-grown tubular structures known as infection threads (ITs), which guide rhizobia transcellularly from root hairs towards the newly formed nodule primordium. The construction of ITs and their progression through cells is tightly controlled by the host plant, which remarkably anticipates their passage by massive reorganization of the cell cytoplasm into a column to direct the path of passage of the growing IT. By combining high-resolution transmission electron microscopy and live cell imaging methods, we have investigated with cellular resolution how IT development and associated cell reprogramming are regulated in the model legume *Medicago truncatula*. These studies provided a comprehensive view of dynamic signalling and ultrastructural cellular changes that occur prior to and during IT construction and progression, and which involve cell-specific calcium responses and novel molecular components that we are currently characterizing. More recent live imaging tools now enable us to address plant-microbial cellular cross-talk in early root hair infection compartments as will be discussed.

Local and systemic regulation of nodulation in *Medicago truncatula*

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Legume plants growing in mineral nitrogen (N) deficit develop a specific root organ in response to rhizobium symbiotic bacteria, the nitrogen-fixing nodule. Long distance (systemic) root-to-shoot-to-root regulatory pathways coordinate the number of nodules formed through signalling peptides perceived by Leucine-Rich Repeats Receptor-Like Kinases. In the *Medicago truncatula* model legume, C-TERMINALLY ENCODED PEPTIDEs (CEPs) are critical to allow root competence for nodulation when mineral N availability is limited, through the COMPACT ROOT ARCHITECTURE 2 (CRA2) receptor acting in shoots[1-2]. To optimize the number of nitrogen-fixing nodules depending on plant needs, another independent systemic pathway involving CLAVATA-LIKE (CLE) peptides and the SUPER NUMERIC NODULE (SUNN) receptor is additionally involved in shoots[3-4]. Furthermore, high levels of mineral N also activate this systemic CLE/SUNN pathway[5]. We showed that these positive and negative systemic pathways are dynamically coordinated 1) in roots by the action of cytokinin hormones acting through the CYTOKININ RESPONSE 1 (CRE1) receptor and the NODULE INCEPTION (NIN transcription factor), leading to the regulation of the production of specific CLE and CEP signalling peptides[6-7]; and 2) in shoots downstream of the CRA2 and SUNN receptor by regulating antagonistically the production of the miR2111 shoot-to-root systemic effector promoting nodulation[5,8]. The NIN-LIKE PROTEIN 1 (NLP1) transcription factor also contributes to the coordinated regulation of specific CLE and CEP peptides depending on N availability[5,9]. Currently, we are interested in 1) integrating other environmental factors modulating the CEP/CRA2 systemic pathway, 2) identifying shoot and root downstream effectors and 3) deciphering how symbiotic-related hormones regulate at the cell-type specific level transcriptional responses leading to nodule formation. Progress on the analysis of some of these symbiotic regulations will be reported.

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PL 11

THE FUN TRANSCRIPTION FACTOR MEDIATES ENVIRONMENTAL CONTROL OF NITROGEN FIXATION

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Plants adapt to fluctuating environmental conditions by adjusting their metabolism and gene expression to maintain fitness. In legumes, nitrogen homeostasis is maintained by balancing nitrogen acquired from soil resources with nitrogen fixation by symbiotic bacteria in root nodules. We identify a novel transcriptional regulator, FIXATION UNDER NITRATE (FUN) that controls nitrogen fixtion in response to the soil environment. Unexpectedly, we identify zinc as a signal connecting the environment with nodule function via FUN. I will discuss the identification, mechanisms of action and implications of FUN regulation for nodulation control and crop improvement.

PL 12

CONTROL OF OXYGEN PERMEATION INTO *LOTUS JAPONICUS* NODULES

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Physiological and morphological adaptations are crucial for protecting the oxygen-labile bacterial nitrogenase enzyme in legume nodules. These adaptations include the production of oxygen-binding leghemoglobin proteins and the formation of an oxygen diffusion barrier in the nodule periphery [1]. However, the molecular mechanisms underlying the formation of this barrier have remained unclear due to the complex interplay between nodule organogenesis, infection, and rhizobia accommodation.

To overcome these challenges, we investigated the phenotypic diversity of *Lotus japonicus* accessions that exhibit a decoupling of nodule organogenesis from nodule infection when inoculated with a sub-compatible *Rhizobium leguminosarum* strain. Through comparative transcriptomics, we identified specific genes associated with oxygen homeostasis and the deposition of lipid polyesters on cell walls that were upregulated in infected nodules compared to uninfected ones. As hydrophobic modifications of cell walls are pivotal for creating diffusion barriers like the root endodermis, we focused on two *Fatty acyl-CoA reductase* (*FAR*) genes that were specifically activated in the root and/or in the nodule endodermis [2]. By studying mutant lines with a defective *FAR* gene, we observed decreased polyester deposition on this cell layer and increased nodule permeability compared to wild-type plants. This led to significantly higher oxygen concentrations in the inner cortex of mutant nodules, resulting in reduced nitrogenase activity and impaired shoot growth [2].

These findings provide compelling genetic evidence for the formation of the nodule oxygen diffusion barrier, a critical adaptation that enables efficient nitrogen fixation in legume nodules.

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PL 13

EVOLUTION OF THE NITROGEN FIXING ROOT NODULE SYMBIOSIS

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To overcome nutrient limitations, plants engage in two main types of root endosymbioses with beneficial microbes. Arbuscular mycorrhiza (AM) is an ancient symbiosis formed by about 70-90 % of land plants and phosphate-acquiring fungi of the phylum Glomeromycota. By contrast, the root nodule symbiosis (RNS) with nitrogen-fixing bacteria is phylogenetically restricted to a single clade encompassing four angiosperm orders – Fabales, Fagales, Cucurbitales and Rosales – within which the distribution of RNS-forming species is scattered[1, 2]. The order Rosales encompasses the economically important family Rosaceae (~3000 species) which comprises four RNS-forming genera that all belong to the Dryadoideae subfamily[3, 4]. From an evolutionary perspective, the genus *Dryas* is particularly interesting because it contains the closely related RNS and non-RNS-forming species *Dryas drummondii* and *Dryas octopetala*, respectively[5, 6, 7]. This genetic polymorphism within the genus *Dryas* holds the potential to discover new genetic element(s) required for the establishment plant root endosymbioses[8]. To identify the causative mutation(s) responsible for the polymorphic symbiosis trait within the *Dryas* genus, we made used of a hybrid species emanating from the cross between *D. drummondii* and *D. octopetala*. We *de novo* sequenced the genomes of *D. drummondii*, *D. octopelata* and the *D. drummondii* × *D. octopelata* hybrid using the PacBio sequencing technology and inspected the ability of the putative F2 population to establish plant root endosymbioses.

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PL 14

MISCANTHUS HOSTS A HIGH DIVERSITY OF DIAZOTROPHIC PGPRs WHEN GROWN IN SCOTTISH SOILS

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Miscanthus (*Miscanthus x giganteous*) is a C4 grass capable of growing in temperate climates. It has low (or zero) fertiliser requirements, and can grow in low fertility soils where it can produce huge amounts of biomass for use as biofuels. Biological N Fixation (BNF) is suggested to be a mechanism allowing *Miscanthus* to grow in low N soils, and it has previously shown to harbour diazotrophs, including *Azospirillum* and *Herbaspirillum* that are more commonly found in tropical C4 grasses like sugarcane[1, 2, 3]. In the present study, *Miscanthus* was established in 3 sites in Scotland: (1) an arable site with sandy loam soils (2) an upland hill farm with humus-iron podzols derived from Old Red Sandstone, and (3) a livestock farm with heavy, poorly drained gleys. A year later the roots were sampled and potential diazotrophic Plant Growth-Promoting Rhizobacteria (PGPR) were isolated using various solid and semi-solid low-N media routinely used for isolation of diazotrophs from plants, such as YMA, TY, NFb and SM. Soil samples for microbiome analysis were also sampled. *Miscanthus* was shown to harbour a very high diversity of culturable bacteria in its roots when grown in various sites in Scotland i.e. >200 isolates were obtained, and the composition of the culturable and non-culturable (microbiome) bacterial communities varied between the sites. Some of the isolates were related to known PGPRs and diazotrophs *e.g. Herbaspirillum, Rhizobium, Azospirillum*. The genomes of two isolates were sequenced; one was a diazotrophic strain of *Herbaspirillum frisingense*, whilst the other was a non-diazotrophic *Herbaspirillum* sp.

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PL 15

LTP AND NITROGEN-FIXING SYMBIOSIS: DISTRIBUTION, EVOLUTION, AND FUNCTION

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Nonspecific lipid transfer proteins (nsLTPs) are secreted antimicrobial peptides, involved in global plant biological processes but also during nitrogen-fixing symbiosis (NFS) established in legumes and actinorhizal symbiosis. Indeed, the nsLTPs are involved at early and nodule steps in both models of NFS. In few legumes (*Medicago truncatula* and *Astragalus sinicus*), some nsLTPs could regulate symbiont invasion, promote root cortex infection, membrane biosynthesis, and improve symbiosis efficiency[1-2]. More recently, a nsLTP named as AgLTP24 has been described in actinorhizal symbioses involving *Alnus glutinosa* and *Frankia alni* ACN14a[3]. AgLTP24 is secreted to deformed root hairs at early step of symbiosis and targeted the symbiont nitrogen-fixing vesicles in nodules. To further study the functions of AgLTP24, the molecular response *F. alni* ACN14a to a sub-inhibitory concentration was conducted using RNAseq. Under these conditions, several genes involved in stress response and nitrogen management were overexpressed by *Frankia*[4]. Although nsLTPs are involved in both models of NFS, their functions and evolutionary history are still largely unknown. Using 15 proteomes of nodulating and non-nodulating plants belonging to all four orders of the RNF clade, phylogenetic analysis was done and showed an independent evolution of nsLTPs in nodulating plants[4]. These results highlighted that nodulating plants co-evolving with their nitrogen-fixing symbionts had independently specialized nsLTPs in symbiosis suggesting a possible convergence of function.

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PL 16

INCREASE: A FOOD LEGUME'S GENETIC RESOURCES INFRASTRUCTURE FOR THE IMPROVEMENT OF BIOLOGICAL NITROGEN FIXATION

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The characterization, maintenance, and exploitation of food-legume genetic resources, to date largely unexploited, form the core development of both sustainable agriculture and a healthy food system. INCREASE is implementing, on chickpea (*Cicer arietinum*), common bean (*Phaseolus vulgaris*), lentil (*Lens culinaris*) and lupin (*Lupinus albus* and *L. mutabilis*), a new approach to conserve, manage and characterize genetic resources (Belucci et al., 2021). The production of industrial fertilizers contributes approximately 3% of global CO2 and is a primary source of the pollutant NO2. Furthermore, runoff from fertilizer is among the world's most serious environmental pollutants, causing also eutrophication of aquatic ecosystems. Therefore, exploiting legume genetic resources to improve the symbiosis between crop legumes and their associated rhizobia could have a major impact on sustainable agriculture and on the world's economic, social, and environmental health. The ability to establish symbiosis with rhizobia, as well as the efficiency of biological nitrogen fixation and competitiveness for nodulation, will be assessed in plant inoculation assays in controlled environmental conditions, whereas validation studies will be performed in order to screen germplasm to identify a set of accessions that could maximize diversity in symbiotic responses based on diversity at candidate genes. In the near future, we plan to establish a collaborative initiative on Biological Nitrogen Fixation, as for other important traits, to maximize the impact of the INCREASE initiative. Here we will thus describe the INCREASE collaborative platform with the aim of inviting all scientists contribute.

INCREASE
Intelligent Collections of Food-Legume Genetic Resources for Agrofood Systems
www.pulsesincrease.eu

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PL 17

INDIGENOUS RHIZOBIA NODULATING GRAIN LEGUMES IN GREEK SOILS AND THEIR IMPACT IN ORGANIC AGRICULTURAL SYSTEMS

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Nitrogen is one of the most rate-limiting nutrients for plant growth in natural and agricultural systems. Although the atmosphere consists of 78% N_2 gas, plants cannot use it. Biological nitrogen fixation (BNF) is the natural process for the conversion of atmospheric N_2 to ammonia (NH_3) and it can be performed by free-living, associative, endophytic and symbiotic rhizobacteria which are collectively called rhizobia and form a symbiotic relationship with legumes. BNF is a key component in sustainable agriculture, since it represents an economic environmentally friendly and renewable nitrogen resource for crops, while the synthetic nitrogen fertilizers have negative effects on the ecosystem and farmers' costs. Therefore, improvement of BNF by selecting suitable combinations of rhizobial strains and legume genotypes will lead to increased productivity and reduced nitrogen (N) fertilizer use. Studies investigating indigenous rhizobia, all over the world, in fields without rhizobial inoculation history are of great importance for selecting novel strains adapted to the local environmental conditions. Such strains often exhibit a better performance in similar habitats and thus they are more preferable for inoculant formulations. To this end, the genetic and symbiotic diversity of indigenous rhizobia nodulating grain legumes (cowpea, common bean and faba bean) in Greek soils were studied[1,2,3,4]. Most isolates were assigned to validly described species, while others constituted novel rhizobial lineages based on multilocus sequence analysis (MLSA). Case studies with selected indigenous rhizobial strains used as inoculants in greenhouse and open field trials will be presented. Legumes inoculated with appropriate rhizobia were used as green manure or intercrops to improve growth, N nutrition and yield of organic greenhouse tomato crop[5,6,7,8]. Inoculation of a soilless hydroponically cultivated common bean with an indigenous rhizobial strain resulted in a substantial reduction of the inorganic-N input, without compromising plant performance[9]. In addition, the indigenous strain was superior to a commercially supplied rhizobial strain in terms of nodulation and BNF capacity, at least for the tested cultivar.

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PL 18

IMPROVED AGRICULTURAL PRODUCTIVITY WHEN DEPLOYING GENE EDITED DIAZOTROPHS ON MILLIONS OF HECTARES OF CEREAL CROPLAND

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We report the development and large-scale adoption of a novel agricultural product based on nitrogen-fixing microbes, demonstrating its efficacy in enhancing corn yields, sustainability, and overall impact on agriculture. Comprehensive analyses of nitrogen fixation assays in vitro and in planta revealed the ability of the microbes to effectively improve nitrogen availability. Field data from millions of hectares showed that corn crops treated with the diazotrophs exhibited higher total nitrogen content, greater yields, and enhanced plant-to-plant yield consistency compared to crops treated with traditional fertilizers. Marked improvements were also observed in nitrogen-limited environments, such as sub-Saharan subsistence farming.

The use of nitrogen-fixing microbes led to reduced nitrate runoff and nitrous oxide emissions, which could translate to significant contributions to a more sustainable agricultural system. Lastly, we observed robust commercial adoption across significant US corn acres, with comparable productivity and economic results to traditional fertilizers. These findings underscore the transformative potential of nitrogen-fixing microbes in revolutionizing how farmers grow the food the world needs while protecting the planet.

PL 19

MEASURING N_2 FIXATION IN POLAR AQUATIC ENVIRONMENTS: NEW INSIGHTS AND CHALLENGES

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N_2 fixation is the primary external source of nitrogen to the Ocean, supporting a significant portion of new primary production in nutrient-poor waters. Estimating global N_2 fixation rates has proven challenging, with uncertainties ranging from less than 100 to over 200 Tg N yr^{-1}. In comparison, oceanic denitrification is estimated to be approximately 400 Tg N yr^{-1}. Other nitrogen sources, such as rivers and atmospheric deposition, are insufficient to balance the nitrogen budget in the Ocean. This suggests that either the Ocean's nitrogen supply is decreasing, which would impact its fertility, or that nitrogen sources are underestimated or sinks are overestimated. To address potential missing nitrogen sources, other ecosystems are explored where N_2 fixation may play a role. Until recently, it was believed that N_2 fixation was primarily carried out by cyanobacteria like *Trichodesmium* in warm tropical or subtropical waters. Traditional assumptions suggested that nitrogen-rich and/or cold waters would not favor N_2 fixation. However, recent studies have expanded our understanding of the habitats and microbial taxa involved in N_2 fixation, including coastal and polar oceans. Here, we present new data from the southern sector of the Indian Ocean, the Antarctic Peninsula and the Arctic Ocean including open and coastal waters. Our results confirm the potential for N_2 fixation in these environments, in particular in coastal shallow waters suggesting an implication of resuspended sediments. We observed higher volumetric N_2 fixation rates in Arctic waters compared to Antarctic waters. Understanding the specific mechanisms driving the observed variations requires more detailed research to link the ecological dynamics to the N_2 fixation patterns of each region. We also emphasize the importance of determining the detection limits of N_2 fixation, especially when measuring low activity but high biomass, as it can potentially result in high false positives.

PL 20

USING NATURAL EXPERIMENTS TO RECONSTRUCT SYMBIOTIC ADAPTATION IN LUCINID CLAMS AND THEIR BACTERIAL PARTNERS

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Studying (co-)evolution in animal-microbial symbioses is challenging due to the differing generation times of animals and bacteria and the inability of typical research projects to replicate the necessary scales of time and space involved in host and microbe adaptation. To overcome these challenges, we are using natural experiments in the sea to reconstruct how symbiotic bacterial partners helped lucinid clams (Lucinidae) adapt to a changing environment. One such natural experiment is the closure of the Isthmus of Panamá, which separated the Caribbean Sea from the Tropical Eastern Pacific about 3 million years ago, resulting in lucinid clams now living in very different habitats on either side of the Isthmus. Using metagenomic sequencing, we characterized the biodiversity and metabolic potential of lucinid symbionts on both sides and found that their genomic functions were very similar, although the potential to fix nitrogen was only found in the Caribbean Sea. Combining our dataset with other symbionts from more than 20 different host species in diverse environments, we show that bacterial symbionts can easily drop and regain nitrogen fixation genes in nitrogen-limited habitats, regardless of phylogeny and host identity. We are now investigating whether nitrogen fixation varies with seasonality and ultimately benefits the host clams.

PL 21

METABOLIC TRADEOFFS CONSTRAIN THE CELL SIZE RATIO IN A MARINE PLANKTONIC NITROGEN-FIXING SYMBIOSIS

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Biological dinitrogen (N_2)-fixation is an important source of N in the environment and is catalyzed exclusively by prokaryotes, some of which are symbionts with multicellular or unicellular eukaryotes[1]. The marine cyanobacteria UCYN-A are unusual N_2-fixing endosymbionts of unicellular haptophyte algae closely related to *Braarudosphaera bigelowii*[2,3]. They depend on each other's carbon (C) and N_2 fixation[2], establishing symbiotic pairs between different UCYN-A lineages and host species that can vary in cell volume up to 1000-fold[3,4,5]. However, whether this natural size variation might inform about the metabolic interdependency of such symbiotic interactions remains unknown. We found that the size ratio between UCYN-A and their algal hosts is strikingly conserved across different lineages/species and metabolic modeling shows that this size relationship maximizes coordinated growth rate based on tradeoffs between nutrient acquisition and exchange. The data also show that size relationships in the UCYN-A symbiosis are similar to those between organelle and cell-size in the eukaryotic plankton, suggesting similar metabolic and growth constraints. These findings suggest that the UCYN-A symbiont is functioning similarly to an organelle, with implications for the evolution of organelles as well as the potential for bioengineering of N_2-fixing plant cells.

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PL 22

THE METABOLIC LANDSCAPE OF *SINORHIZOBIUM MELILOTI*

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Rhizobia have large genomes encoding for a diverse array of metabolic capabilities that help them adapt to varied environmental conditions. Understanding rhizobial fitness in diverse environments, or during symbiosis, requires developing a holistic picture of how various metabolic pathways integrate and collectively contribute to fitness. To this end, over the past several years we have used the functional genomics technique of transposon-sequencing[1] and the *in silico* approach of metabolic modelling[1-3], to generate a systems-level understanding of the metabolism and genetics of the model rhizobium species *Sinorhizobium meliloti*. Here, I will describe our ongoing studies using transposon-sequencing to uncover genes contributing to the fitness of *S. meliloti* across a broad range of nutritional and stress conditions, and the use of metabolic models to interpret the results in a consistent, systems-level framework. These results will provide foundational knowledge about rhizobial metabolism, and they will assist in identifying functions for genes currently of unknown function.

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PL 23

SYSTEMS GENETICS OF MUTUALISTIC PARTNER QUALITY IN LEGUME-RHIZOBIUM SYMBIOSIS

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We live in a symbiotic world. Addressing the challenges of our time, from sustainable agricultural production to ecosystem resilience in the face of climate change, is only possible by fully incorporating symbiotic microorganisms into our biological understanding. Plant symbionts have a broad range of effects on their partners, which percolate up from genomic variation through expression and metabolic networks to result in complex traits like nitrogen fixation, plant growth, and fitness. The mechanistic basis of these benefits, and how they evolve in nature, is key to a predictive and thus useful understanding of symbiosis. Here we integrate genomic, phenotypic, and gene expression data to associate novel sets of horizontally-transferred gene clusters in natural populations of a model symbiont with plant growth, revealing novel genetic processes with important implications for both the function and inheritance of symbiosis function in this important interaction.

ORAL PRESENTATION

ORAL PRESENTATION

O 1

ENGINEERING N_2-FIXATION BY RHIZOBIA ON CEREAL ROOTS

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The root microbiota is critical for agricultural yield, with growth-promoting bacteria able to solubilise phosphate, produce plant growth hormones, antagonise pathogens and fix N_2. Plants control the microorganisms in their immediate environment and this is at least in part through direct selection, the immune system, and interactions with other microorganisms. Considering the importance of the root microbiota for crop yields it is attractive to artificially regulate this environment to optimise agricultural productivity. Towards this aim we developed a synthetic pathway for the production of the rhizopine *scyllo*-inosamine in plants(1). We demonstrated the production of this bacterial derived signal in both *Medicago truncatula* and barley and showed its perception by rhizosphere bacteria, containing a novel biosensor. We have recently developed a hybrid rhizopine uptake system that conveys upon our model strain *Azorhizobium caulinodans* ORS571 ~10^3-fold improved sensitivity for rhizopine perception(2). Using this improved genetic circuitry, we engineered rhizopine-dependent induction of a mutant *nifA*. As the global regulator of nitrogenase genes we showed that rhizopine controlled NifA drives N_2 fixation that partially escapes feedback inhibition by ammonium both *in vitro* and by bacteria colonizing barley roots(3). Most recently we have engineered bacteria to secrete ammonia by controlling the adenylylation state of glutamine synthetase, allowing secretion of large amounts of ammonia. In addition, we have isolated flavonoid independent variants of NodD, enabling rhizopine to induce lipochitooligosaccharide synthesis and hence plant responses. Thus, we have engineered two-way communication between rhizobia and cereal plants, which is a significant step in the engineering of N_2 fixation in cereals.

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ORAL PRESENTATION

O 2

A FRIENDLY DIALOGUE BETWEEN PLANTS AND BENEFICIAL MICROBES-LIPOCHITOOLIGOSACCHARIDE PERCEPTION IN CEREALS

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To achieve sustainable productivity in agriculture, it is necessary to reduce the global dependence on inorganic fertilizers. This can be partially accomplished through better use of beneficial microbial associations, such as engineering biological nitrogen-fixation into non-legume cereal crops. Beneficial plant-microbe interactions rely on symbiosis signaling and here we show that cereals can perceive lipochitooligosaccharides (LCOs) for activation of symbiosis signaling, surprisingly including Nod factors produced by nitrogen-fixing bacteria. Different with legumes which strongly discriminate between LCOs produced by its symbiotic and non-symbiotic bacteria, cereals show non-stringent perception of LCOs. We further discover that LCO perception in both legumes and cereals is activated by nutrient starvation, through transcriptional regulation of *Nodulation Signaling Pathway* (*NSP*)*1* and *NSP2*. These transcription factors regulate strigolactone biosynthesis and act through the karrikin-like receptor DWARF14-LIKE pathway to control the expression of a LCO receptor, which is necessary for LCO perception. Consistently, either overexpression of *NSP2* or pretreatment of strigolactones and karrikins enhances LCO perception in plants. In this work, we demonstrate the ability of cereals to recognize LCOs produced by nitrogen-fixing bacteria, as well as a mechanism of nutrient regulation of LCO perception in legumes and cereals. Our work has implications for generating nitrogen-fixing cereals and delivering more sustainable crop production.

ORAL PRESENTATION

O 3

CHARACTERIZATION OF THE RUBISCO-LIKE PROTEIN IN RHIZOBIAL STRAINS

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Carbon fixation is an important process because carboxylation occurs in several central reactions. Some carboxylating enzymes require a cofactor such as the vitamin biotin. In some rhizobial strains it is crucial since they can not synthesize biotin. We previously reported the phenotypic changes of such auxotrophic rhizobia influenced by the progressive lack of biotin: growth declining, poly beta hydroxybutyrate accumulation, and activity reduction of pyruvate carboxylase, pyruvate dehydrogenase or malate dehydrogenase [1]. The addition of biotin helped to recover the growth and activities. We found that other factors can also participate independently in growth recovery, for example aspartic acid analogs or excess air [2]. The recovery in latter cases depended on the presence of a distant ribulose bisphosphate carboxylase (Rubisco) homolog, Rubisco-like protein (RLP). Is RLP participating directly in carboxylation? In order to answer this question, we are performing experiments to characterize this novel trait in *Rhizobium etli* CFN42 and *R. phaseoli* CIAT652 strains.

Assays include radiactive carbon fixation with living cells and Rubisco enzymatic activity in crude extracts. Inhibitors and activators are also being used. Wild-type and mutant strains are used for comparison, as also a strain that overexpresses the gene *rlp* (XRLP). In XRLP strain extracts, we found a Rubisco activity several times higher than that of wild-type. Enzyme activators were sodium bicarbonate, ATP and ribulose bisphosphate. Hydroxylamine was found as inhibitor. Additionally, we are studying the transcriptional regulation of *rlp* gene through a *lacZ* fusion and the phenotype of the mutants in symbiosis with common bean (*Phaseolus vulgaris*). Further estudies will include the purification of the enzyme.

To determine the carboxylation capability of rhizobial RLP will reveal a new pathway of this important process.

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ORAL PRESENTATION

O 4

MECHANISM FOR THE COWN-MEDIATED PROTECTION OF NITROGENASE AGAINST CARBON MONOXIDE INHIBITION

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Nitrogenase is tightly inhibited by carbon monoxide (CO). To prevent CO inhibition, nitrogen fixing bacteria (diazotrophs) express a protein called CowN that protects nitrogenase from CO. The mechanism of CowN protection is unknown and was proposed to be similar to protection from dioxygen (O_2) by an FeSII protein (Shethna protein), which shuts down all nitrogenase turnover. This presentation discusses the mechanism of CO protection by CowN. We show that CowN and nitrogenase interact and that CowN decreases the affinity of CO binding to FeMoco, however, there is no evidence that CowN directly alters the reactivity of FeMoco. CowN does not turn off nitrogenase turnover, indicating that protection against CO and O_2 inhibition occur through distinct mechanisms. CowN is composed of a small 4-helical bundle. Mutational analysis identified a critical C-terminal glutamic acid residue that mediates protein-protein interaction with nitrogenase and is required for CowN function. Crosslinking experiments between CowN and MoFeP detected at least two CowN binding sites on MoFeP, one on the α-subunit and another on the β-subunit. Both binding sites contain a surface cysteine residue. Taken together, our results suggest that CowN operates by preventing CO access to the MoFeP active site, while allowing dinitrogen reduction to continue. The functional reason for the dual CowN binding sites is still under investigation. In addition to determining how nitrogenase is protected from CO inhibition, this work demonstrates that nitrogenase's substrate specificity can be tuned through protein-protein interaction. Furthermore, it illustrates how the three nitrogenases (Mo, V, and all Fe) circumvent CO inhibition using different mechanisms.

ORAL PRESENTATION

O 5

CRYO-ELECTRON MICROSCOPY OF NITROGENASE – FESII COMPLEX

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Nitrogenase is irreversibly inactivated by oxygen. The Fe protein has a half-life in air of ~1 min while MoFe is stable for minutes to hours [(1)]. Diazotrophs have evolved several mechanisms for protecting nitrogenase proteins from oxygen. The obligate aerobe *Azotobacter vinelandii* is notable for its ability to carry out nitrogen fixation in super-atmospheric concentrations of oxygen. Intracellular oxygen concentration is controlled by diffusion barriers and through very high respiration rates. *Azotobacter* also has the ability to temporarily and reversibly shield Mo-Nitrogenase from oxygen through the formation of a catalytically inactive ternary complex with the ferredoxin FeSII, which is called conformational protection [(2, 3, 4)]. The crystal structure of FeSII was solved in this lab (PDB 6YAV) and by Schlesier et al. [(4)]. The current working model of conformational protection is the redox-switch model, whereby a large conformational change induced by the oxidation of it's 2Fe-2S cluster activates FeSII for binding in a crevice between Fe and MoFe [(4)]. While the ternary complex has been partially purified [(2, 3, 4)], owing to its low natural abundance, fragility and limited oxygen tolerance the structure has not been solved. We have purified the ternary complex of Mo-Nitrogenase and FeSII to homogeneity by reconstitution from the component proteins and anaerobic SEC. We have begun with the mechanistic and biophysical characterisation of this complex and drawing on recent advancements in electron microscopy of nitrogenase proteins, we are now optimising anaerobic grid freezing for structure determination by cryo-EM. To further investigate the function of FeSII we are also studying mutants and homologs of FeSII and recently solved the crystal structure of an FeSII homologue from *Azotobacter beijerinckii*.

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ORAL PRESENTATION

O 6

UNDERSTANDING AND OPTIMIZING TARGETED NITROGENASE EXPRESSION IN GAMMAPROTEOBACTERIA FOR SUSTAINABLE CEREAL AGRICULTURE

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A major aim of biological research in the 21st century is to expand the use of effective biological nitrogen fixation to cereal crops, thereby reducing our dependence on exogenous nitrogen fertilizer. Diazotrophic Gammaproteobacteria have the potential to serve as root-associated nitrogen fixers and offer a promising avenue for achieving this goal[1]. Among these, *Kosakonia radicincitans* DSM16656 (KrDSM16656), is a candidate of significant interest, as it is a N_2-fixing endophyte, an efficient root colonizer, and has been shown to promote plant growth through various mechanisms[2]. In this study we aim to understand the regulation of nitrogenase activity in KrDSM16656, focusing on the influence of carbon and nitrogen availability, and exploring alterations that enable it to be more efficient and targeted. High-throughput and multi-omics approaches are used to explore these effects, along with root colonization on the model cereal plant barley. This helps guide engineering approaches that establish nitrogen fixation in response to plant derived signals, thus ensuring targeted nitrogenase expression and enabling the strain to remain a competitive colonizer under field conditions. Mutants of the nitrogenase regulator operon, *nifLA,* were made and verified through acetylene reduction assays. These mutants provide a chassis for taking control of nitrogen fixation in KrDSM16656 and open up the possibility of coordinating it with root-associated signals. RNA-Seq has allowed for suitable promoters for root-associated expression to be identified, further verified through promoter-reporter fusions coupled with flow-cytometry. The same approach was applied, in tandem, to the genetically related bacteria *Enterobacter ludwigii* AA4 (ElAA4)[3], to explore the potential of transferring the nitrogen fixation clusters from KrDSM16656 to ElAA4. ElAA4 exhibits robust colonization capabilities and lacks undesirable regulatory components, making it an ideal candidate for further exploration. Overall, this work helps shed light on the resource allocation required for nitrogen fixation and provides insights that enhance and inform rational engineering strategies for sustainable cereal agriculture.

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ORAL PRESENTATION

O 7

BIOELECTROCHEMICAL NITROGEN FIXATION DRIVEN BY CATHODIC BIOFILMS

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The electrotrophic production of bacterial biomass represents a challenging opportunity with several industrial applications in terms of organic carbon storage and soil fertility improvement. To evaluate biomass development driven by electroactive microorganisms, an environmental microbial consortium was inoculated in a bio-electrochemical system (BES) where only N_2 and inorganic carbon were provided. The experimental set-up included three BESs inoculated with microbial consortium and operated at a constant cathodic potential of −0.7 V *vs* SHE. Those were compared with four control systems: one BES inoculated with microbial consortium and operated at a constant cathodic potential of −0.2 V *vs* SHE; one BES not inoculated and operated at a constant cathodic potential of −0.7 V *vs* SHE; two BESs inoculated with microbial consortium and operated at open circuit (OC). After an initial period of stabilization, all seven BESs were run for 165 days.

A higher charge consumption was observed in the inoculated systems at -0.7 V polarization compared with the controls. At the end of incubation, polarized inoculated systems produced a higher biomass in the cathodic chamber compared with OC. DNA and RNA were isolated from the cathodic biofilm, the microbial suspension and the bottle wall biofilm and microbial consortia are under characterization by Illumina 16S rRNA gene sequencing and by shotgun sequencing.

These outcomes offer new insights on the ecological and physiological mechanisms involved in the establishment of microbial consortia in oligotrophic environments, and pave the way for novel biotechnological applications.

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ORAL PRESENTATION

O 8

UMAMIT AMINO ACID TRANSPORTERS AND THEIR ROLE IN LEGUME NODULATION

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During root nodulation both the rhizobial symbiont and the legume host must coordinate resources to ensure proper growth and maintenance of the symbiotic relationship. To determine if the provision of amino acids across symbiotic membranes is required to establish and maintain symbiosis, we examined the role of the Usually Multiple Amino acids Move In and out Transporter (UmamiT) family[1]. In addition to a previously published MtUmamiT14[2], we identified several *Medicago truncatula UmamiTs* that show strong symbiosis-associated expression[3,4,5], which are candidates for either rhizobial feeding or assimilate transport. Using transcriptome analysis we showed that UmamiT nodulins 1, 2, & 6 (*UTN1/2/6*) are expressed in root hairs[4] within hours of inoculation[5] indicating a likely role in rhizobial feeding during the infection stages. UTN promoter:GUS constructs suggested that UTN1, 2, 3, & 6 localised to infection associated zones and internal vasculature in mature nodules. Phylogenetic analysis indicated that UTN1 and UTN2 are distinct from UTN3-7, which appear to be part of a large legume-specific gene radiation covering both determinate and indeterminate nodulators. Many of the UTN orthologues in other legumes were similarly expressed throughout nodulation[6,7,8] indicating a likely conserved function across legumes. Amino acid transport was confirmed for UTN1 and UTN2 using the *Xenopus laevis* oocyte heterologous expression system. To determine the importance of these symbiosis-associated UmamiTs, we generated UTN1/2/6 triple mutants via CRISPR, which show an impaired infection phenotype indicating the action of these UTNs is important to the establishment of infection. Further work is necessary to identify complete substrate capacity and subcellular localisation, as well as the effect of these symbiosis-associated UTNs to nitrogen provision to the plant as a whole.

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ORAL PRESENTATION

O 9

LYSM RECEPTORS HAVE A PROGRAMABLE CAPACITY FOR LIGAND PERCEPTION AND DOWNSTREAM SIGNALLING ENABLING RATIONAL ENGINEERING

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Receptor-like kinases (RLKs) are watchmen “on duty” at the membrane of plant and metazoan cells. They recognize a signal, interpret it, and initiate a corresponding intracellular response. In plants, RLKs control plant development and responses to symbiotic or pathogenic microbes. LysM RLKs are plant inventions that emerged early during their evolution and enable the perception of glycan ligands of microbial origin. Importantly, these receptors are able to distinguish between beneficial and pathogenic types of glycans and mount a symbiotic or immune response, accordingly. A key receptor present in all plants is CERK which enables the recognition of chitin (CO6-8) released by pathogenic fungi and the activation of immunity. Legume plants evolved Nod factor receptors (NFR) that recognize lipochitooligosaccharides (LCOs) with high specificity and sensitivity enabling symbiosis with nitrogen-fixing bacteria. We have identified how legume LysM receptors evolved specific LCO perception and how chitinous signals are distinguished at the molecular level is currently unknown. Next, we examined the functional role and biochemical properties of chimeric CERK6-NFR1 receptors and identified distinct signatures in the intracellular regions to be required and sufficient for determining immunity and symbiotic signalling in Lotus roots. Our findings provide a molecular framework for deciphering specific recognition of COs in plants and for engineering downstream signalling leading to root nodule symbiosis.

ORAL PRESENTATION

O 10

DIVERSE TYPE 3 EFFECTORS CAN TRIGGER NODULATION INDEPENDENTLY OF NOD FACTORS

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Some *Bradyrhizobia* have the original ability to nodulate certain legume species, such as *Aeschynomene* spp. or *Glycine max*, independently of Nod factors (NFs). Two NFs-independent symbiotic processes that differs by the requirement or not of the type III secretion system (T3SS) were described[1,2]. ErnA is the first type 3 effector (T3E) shown to trigger nodulation in *Aeschynomene indica*[3]. To better understand these alternative symbioses, the symbiotic properties of a large collection of 196 *Bradyrhizobium* strains were analyzed on *A. indica*. We focused on strains whose genome was sequenced to establish correlations between their genomic content and their symbiotic properties. Through a combination of comparative genomic analyses and mutagenesis of some candidate genes emerging from these analyses, we identified divers T3Es triggering nodulation in *A. indica*. During this talk, I will present some characteristics of these T3Es and additional insights concerning the ability of *Bradyrhizobia* to nodulate some legumes independently of NFs.

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ORAL PRESENTATION

O 11

NANOBODY-DRIVEN SIGNALING REVEALS THE CORE RECEPTOR COMPLEX IN ROOT NODULE SYMBIOSIS

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The two *Lotus japonicus* Nod factor receptors NFR1 and NFR5 have long been shown to recognize lipochitooligosaccharides and initiate root nodule organogenesis and bacterial infection. However, little was known about how NFR1 and NFR5 interact to activate downstream signaling. To address this, we developed a synthetic approach based on nanobodies to drive the assembly of the two receptors[1]. We show that NFR1 and NFR5 constitute the core receptor complex initiating the cortical root nodule organogenesis program as well as the epidermal program controlling infection[1]. We find that organogenesis signaling is mediated by the intracellular kinase domains whereas infection requires functional ectodomains. Finally, we identify evolutionarily distant barley receptors that activate root nodule organogenesis, which could enable engineering of biological nitrogen-fixation into cereals[1].

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ORAL PRESENTATION

O 12

TRACKING *SINORHIZOBIUM MELILOTI* CELL PROLIFERATION DYNAMICS DURING EARLY STAGES OF HOST INVASION

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Rhizobia enter into symbiosis with legumes in specialized root organs called nodules where they fix nitrogen for their host in exchange for carbon substrates. Establishing this interaction requires precise molecular exchanges between both partners before rhizobia can colonize developing nodules, which they invade in most cases *via* a tunnel-like compartment formed in root hairs called the infection thread (IT). While the genetic reprogramming of rhizobia has been well studied in nodules[1], very little is known about the bacterial reprogramming occurring during early root infection. We have generated new molecular tools, which enable highly sensitive tracking of rhizobial (*Sinorhizobium meliloti*) promoter activities and protein accumulation and localization patterns within ITs of model legume *Medicago truncatula* using fluorescent confocal microscopy. We are using these tools to address how rhizobia reprogram in the early root hair compartments, specifically in terms of cell proliferation. Using cell cycle markers, we show that the unipolar growth mode characteristic of *S. meliloti* cells is maintained *in planta*. We provide further evidence that bacterial cell proliferation is spatially and temporally dynamic in early infection compartments[2]. Since bacterial cell proliferation has been proposed as a means for the progression of nodule colonization, tracking its dynamics in early infection compartments could provide new insights into the form of motility used by rhizobia during host invasion.

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ORAL PRESENTATION

O 13

AN EVOLUTIONARILY SHARED FORMIN PROTEIN MEDIATES DIFFERENT SYMBIOTIC INTRACELLULAR INFECTIONS BY SPECIFIC TRANSCRIPTION GATING IN LEGUME AND NON-LEGUME PLANTS

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Symbiotic infections own specific cellular inventions to entrap the symbionts to allow intracellular infection. This whole process involves stepwise and distinct actin cytoskeleton dynamics. Here, we identified a novel actin nucleator gene *INFO*, which is required for cytoskeleton remodeling at the onset of rhizobia and AMF intracellular entry in *Medicago truncatula.* Rhizobia infections on *info* mutant roots are mostly blocked at the stage of IT initiation. INFO protein itself localizes to the plasma membrane but undergoes a pattern transition from a homogenous distribution in the absence of rhizobia to punctate confinement in SYMREM1-positive nanodomains upon rhizobia inoculation. FLIM-FRET analysis supports their physical interaction *in vivo*. Additionally, the INFO cytosolic domain FH1FH2 could bind G-actin with high affinity (Kd=825.23 nM). Given that INFO is evolutionary not restricted to the nodulating clade but well confined in AM-forming species, a specific correlation between INFO promoter activation and AMF infection is further confirmed. Here the protein localizes to the cell periphery of cells hosting freshly branched arbuscules. In line with this, AMF colonization is compromised in *info* mutants.

Transcriptionally, *INFO* is upregulated upon both infections. EMSA and transactivation assays suggest *INFO* is upregulated and targeted by NIN. Notably, its tomato orthologue *SlINFO* could also be activated by NIN despite NIN being lost in non-legume plants. Interestingly, the upstream component of NIN, CYCLOPS, could bypass the transcription factor activation route and directly activate the expression of *SlINFO* but not *MtINFO*. This unconventional hierarchical transcriptional gating network sheds light on the engineering potential of rhizobia infections in non-legumes.

ORAL PRESENTATION

O 14

MILDEW LOCUS O (MLO) PROTEINS ARE REQUIRED FOR RHIZOBIAL INFECTION IN *MEDICAGO TRUNCATULA*

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Mildew Locus O (MLO) proteins, named after the robust resistance of the barley *mlo* mutant to powdery mildew, are involved in many processes, including reproduction, thigmotropism, and biotrophic interactions with biotrophic fungi, which besides powdery mildew, also include the endophyte *Serindipita indica*[1], and arbuscular mycorrhizal fungi[2]. Their biochemical role remained a mystery for many years, but recent studies have revealed they act as calcium channels. We have identified a root-hair expressed MLO gene that when mutated strongly decreases rhizobial infection. A second root-hair expressed MLO gene, which encodes the ortholog of Arabidopsis NORTIA which is involved in reproduction, was found to be induced by rhizobia and Nod factors. A double mutant showed defects in infection and a decrease in nodule number. Transcript profiling, along with genetic and physiological studies, have provided insight into the underlying mechanism. Given that every biological process linked to MLOs so far involves contact with an external stimulus, we propose that MLOs are involved in sensing rhizobial attachment to direct infection thread growth.

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ORAL PRESENTATION

O 15

RINRK1 PROMOTES ACCUMULATION OF NOD FACTOR RECEPTORS IN NANODOMAINS ENHANCING RHIZOBIAL INFECTION OF LEGUME ROOTS

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During legume-rhizobia root-nodule symbioses, rhizobial Nod factor (NF) signals are recognized by NF receptors and activate both nodule organogenesis in the root cortex and a program leading to infection in the root epidermis. NF receptors' intracellular kinase domains are sufficient to mediate signaling to promote organogenesis whereas infection requires functional ectodomains [1]. We demonstrated that *RinRK1* was induced by NF signaling and is required for formation of infection threads to enable rhizobial infection [2]. Here we show that RinRK1 interacts with the extracellular domains of NF receptors NFR1 and NFR5 to promote their accumulation at root hair tips in response to NFs. In parallel, we found that the nanodomain-protein Flotillin 1 (Flot1) associates with the kinase domains of NFR1, NFR5 and RinRK1 to promote the accumulation of NFR1 and NFR5 at root hair tips in response to NFs, and that RinRK1 promotes Flot1 interactions with NFR1 and NFR5. We conclude that RinRK1 serves as a scaffold to facilitate interactions between NF receptors and Flot1 to promote their accumulation in PM nanodomains at root hair tips, resulting in enhanced NF signaling that promotes formation of infection threads.

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ORAL PRESENTATION

O 16

AERLCK2, A RECEPTOR LIKE CYTOPLASMIC KINASE REQUIRED FOR NOD-INDEPENDENT RHIZOBIAL SYMBIOSIS IN *AESCHYNOMENE EVENIA*

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The agronomic and ecologic interest of legumes stems from their capacity to establish a nitrogen-fixing symbiosis with rhizobia, which they host within their nodules. If the majority of leguminous plants resort to a widespread and sophisticated symbiotic mechanism, some species deploy alternative processes hitherto little studied. Among them, some species from the genus *Aeschynomene* present an original symbiotic system, since they are able to nodulate with some photosynthetic *Bradyrhizobium* independently of the perception of rhizobium Nod factors and the formation of infection threads.

To update the molecular mechanisms involved in this Nod-independent symbiosis, a genetic screen was conducted and allowed the identification of 250 nodulation mutants of the model plant *Aeschynomene evenia* [1]. The sequencing of mutants altered in the earliest stages of nodulation led notably to the identification of a new symbiotic gene, *AeRLCK2*, putatively coding for a Receptor-Like Cytoplasmic Kinase (RLCK). RLCKs are proteins known to be involved in the early stages of many signaling pathways in plants by interacting with ligand-binding Receptor-Like Kinases (RLKs) and relaying the signal perception intracellularly. To reach a better understanding of *AeRLCK2*'s symbiotic role, phenotypic and molecular characterisation of *rlck2* mutants were carried out. We show here that *AeRLCK2* is required for nodule initiation and is essential for the activation of the symbiotic signaling pathway. To go further, we are now using biochemical approaches to test if RLCK2 is able to interact with other key signaling actors to mediate *Bradyrhizobium* perception, signal transduction and/or infection.

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ORAL PRESENTATION

O 17

CHROMATIN REGULATORS ASSOCIATED WITH THE PIONEER TRANSCRIPTION FACTOR NFYA1, DURING NODULATION OF *MEDICAGO TRUNCATULA*

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MtNF-YA1 is a nodule specific CCAAT box binding transcription factor, which regulates both rhizobial infection and nodule development in legumes [1, 2, 3]. NF-Y functions as a pioneer transcription factor, which promotes chromatin accessibility for cell-type specific master transcription factor in both plants and animals [4,5]. However, whether NF-Y is able to modulate chromatin accessibility by itself or by recruiting other chromatin remodelers remains elusive. Using a proximity-dependent biotynilation (PDB) approach [6], we identified several NF-YA1 interactors with chromatin modification potential including some members of the Argonaute family (AGO). Beside their well-known function as cytoplasmic effectors of small RNA-mediated transcriptional and post-transcriptional silencing pathways, AGO proteins have also recently been found to act as chromatin-based promoters of gene transcription [7]. Interestingly, in common bean and soybean, Ago5 plays essential roles in the establishment of the symbiosis with rhizobia [7,8]. *in planta* interaction studies and functional studies of the symbiotic role of these NF-YA1 interactors will be presented and discussed.

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ORAL PRESENTATION

O 18

SUGAR SIGNALING ACTS AS A PROXY FOR CYTOKININ SIGNALING FOR DE NOVO MERISTEM FORMATION DURING NODULE ORGANOGENESIS

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Symbiosis between plants and diazotrophs require formation of a *de novo* meristem for endocytic accommodation of symbionts, a process that is tightly regulated by plant hormones cytokinin and auxin. Cytokinin signaling through CRE1 receptor causes auxin accumulation by regulating its transport or biosynthesis to initiate cell division for nodule organogenesis. Accordingly CRE1 mutant (*cre1*) is unable to undertake symbiosis and our objective was to strategize and restore functional symbiosis in *cre1* for understanding the downstream events. We show that sucrose as well as turanose (non-metabolizable sucrose) treatment can recover functional symbiosis in *cre1* indicating the importance of downstream sucrose signaling in symbiosis [1]. An auxin conjugate hydrolase *MtIAR33* was highly upregulated by sucrose signaling. Overexpression of *MtIAR33* could restore symbiosis in *cre1* and its knockdown reduced nodule number in A17 indicating deconjugation of auxin conjugates to be a potential pathway of auxin accumulation during nodule organogenesis [1]. Additionally sugar signaling significantly upregulated an auxin responsive homeobox transcription factor WOX5 well known for its role in meristem maintenance. This prompted us to check whether overexpression of *WOX5* could also rescue *cre1*. Intriguingly *MtWOX5* from *Medicago truncatula* having indeterminate nodule meristem failed to rescue *cre1* but *AhWOX5* from *Arachis hypogaea* having determinate meristem could completely rescue *cre1*[2]. We have shown that *MtWOX5* but not *AhWOX5* function as a repressor and swapping a single amino acid is sufficient to convert *MtWOX5* to *AhWOX5* function and vice versa.

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ORAL PRESENTATION

O 19

A RARE NON-CANONICAL SPLICE SITE IN *TREMA ORIENTALIS SYMRK* DOES NOT AFFECT ITS DUAL SYMBIOTIC FUNCTIONING IN ENDOMYCORRHIZA AND RHIZOBIUM NODULATION

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Nodulating plants share a conserved set of symbiosis genes, whereas related non-nodulating sister species show pseudogenization of several key nodulation-specific genes[1,2]. Signalling and cellular mechanisms critical for nodulation have been co-opted from the more ancient plant-fungal arbuscular endomycorrhizal symbiosis[3]. Studies in legumes and actinorhizal plants uncovered a key component in symbiotic signalling, the LRR-type SYMBIOSIS RECEPTOR KINASE (SYMRK), which is essential for nodulation and arbuscular endomycorrhizal symbiosis[4]. To our surprise, however, despite its arbuscular endomycorrhizal symbiosis capacities, we observed a seemingly critical mutation in a 5'-intron donor splice site in the *SYMRK* gene of *Trema orientalis,* the non-nodulating sister species of *Parasponia*. This led us to investigate the symbiotic functioning of *SYMRK* in the *Trema-Parasponia* lineage and to address the question to what extent a single nucleotide polymorphism in a 5'-intron donor splice site affects the symbiotic functioning of *SYMRK*. CRISPR-Cas9 mutagenesis confirmed that *SYMRK* is also essential for nodulation and endomycorrhization in *Parasponia andersonii*. Subsequently, it is revealed that the 5'-intron donor splice site of *SYMRK* intron 12 is variable and, in most dicotyledon species, doesn't contain the canonical dinucleotide 'GT' signature but the much less common motif 'GC'. Strikingly, in *T. orientalis,* this motif converted into a rare non-canonical 5'-intron donor splice site 'GA'. This *SYMRK* allele, however, is fully functional and spreads in the *T. orientalis* population in Malaysian Borneo. A further investigation into the occurrence of the non-canonical GA-AG splice sites confirmed that these splice sites are extremely rare. In conclusion, the discovery of this functional GA-AG splice site in *SYMRK* highlights a gap in our understanding of splice donor sites.

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ORAL PRESENTATION

O 20

IDENTIFICATION OF TYPE 3 SECRETION SYSTEM EFFECTORS IMPACTING *RHIZOBIUM*-LEGUME SYMBIOSIS IN *VIGNA* SPECIES USING THE *BRADYRHIZOBIUM VIGNAE* STRAIN ORS3257

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In plant-bacteria interactions, a Type 3 secretion system (T3SS) has been shown to establish an alternative symbiotic process between bacteria and legumes. This study focused on understanding how T3SS impacts the rhizobium-legume symbiosis in *Vigna* species that are important crops and protein sources in Asia and Africa. The elite *Bradyrhizobium vignae* strain ORS3257 (recommended for cowpea inoculation in Senegal) was used to identify key effectors that either promote symbiotic efficiency or render the interaction incompatible. The results revealed that the T3SS had a significant positive impact on the symbiotic efficiency of the ORS3257 in *V. unguiculata* and *V. mungo*, while it completely blocked symbiosis with *V. radiata*. The identification of the T3 effectors (T3Es) responsible for the symbiotic interaction of ORS3257 with these three *Vigna* species indicated that the $NopP_2$ effector compromised symbiosis with *V. radiata*, while at least four effectors (NopT, NopAB, $NopM_1$, and $NopP_1$) positively impacted on symbiotic interaction with *V. mungo* and *V. unguiculata*.

Identifying key effectors is crucial to improve the growth of *Vigna* species in Africa and Asia. T3Es vary from strain to strain, so studying a diverse range of strains is necessary to understand the rules governing symbiosis. This study's findings offer insights into using the T3SS to enhance crop growth in Asia and Africa. Further research could explore using other bacterial strains and effectors to improve symbiosis, leading to sustainable agriculture and increased food security in these regions.

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ORAL PRESENTATION

O 21

WIDELY CONSERVED AHL TRANSCRIPTION FACTORS ARE ESSENTIAL FOR *NCR* GENE EXPRESSION AND NODULE DEVELOPMENT IN *MEDICAGO*

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Symbiotic nitrogen fixation by rhizobium bacteria within the cells of legume root nodules alleviates the need for nitrogen fertilizers. Nitrogen fixation requires the endosymbionts to differentiate into bacteroids which can be reversible or terminal[1]. The latter is controlled by the plant, it is more beneficial and has evolved in multiple clades of the Leguminosae family. The plant effectors of terminal differentiation in IRLC legumes are Nodule-specific Cysteine-Rich NCR peptides[2], which are absent in legumes such as soybean where there is no terminal differentiation of rhizobia. It was assumed that *NCR*s coevolved with specific transcription factors, but our work demonstrates that expression of *NCR* genes does not require *NCR*-specific transcription factors[3]. Introduction of the *Medicago truncatula NCR169* gene under its own promoter into soybean roots resulted in its nodule-specific expression, leading to bacteroid changes associated with terminal differentiation. We identified two AT-Hook Motif Nuclear Localized (AHL) transcription factors from both *M. truncatula* and soybean nodules that bound to AT-rich sequences in the *NCR169* promoter inducing its expression. Whereas mutation of *NCR169* arrested bacteroid development at a late stage, the absence of MtAHL1 or MtAHL2 completely blocked bacteroid differentiation indicating that they also regulate other *NCR* genes required for development of nitrogen-fixing nodules. Regulation of *NCR*s by orthologous transcription factors in non-IRLC legumes opens the possibility of increasing the efficiency of nitrogen fixation in legumes lacking *NCR*s.

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ORAL PRESENTATION

O 22

CHROMATIN REMODELING IN A SUBSET OF EPIDERMAL CELLS DURING ROOT NODULE SYMBIOSIS

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Transcription factors (TFs) are crucial for triggering specific cellular responses through the regulation of downstream genes. As for root nodule symbiosis (RNS), CYCLOPS and NIN are two key TFs that are essential for infection of rhizobia in the root epidermis and development of root nodules in the root cortex. Phylogenetic analysis has revealed that the gain of *cis*-regulatory elements (CREs) bound by these TFs are highly correlated with the evolution of RNS. Chromatin remodeling that allows access to appropriate CREs by the TFs is thought to be a prerequisite for a regulatory response. So, we carried out single-cell ATAC-seq of *Lotus japonicus* roots to investigate the initial chromatin remodeling of host plant cells against rhizobia. Single-cell analysis is especially useful to dissect cell type-specific responses, which may widely differ between the epidermis and the cortex. We detected an epidermal cell-specific chromatin remodeling in response to inoculation of compatible microsymbiont, *Mesorhizobium loti*. Among these cells, we further found enrichment of a specific motif among the uniquely accessible loci, including the promoter regions of *ERN1* and *NIN*, that is potentially targeted by CYCLOPS. This indicates that CYCLOPS plays a major role in regulation of gene expression among these epidermal cells that is necessary for successful infection of rhizobia.

ORAL PRESENTATION

O 23

EPIGENETIC MECHANISMS REGULATE TRANSCRIPTION OF SYMBIOTIC ISLANDS DURING NODULE DEVELOPMENT

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A large fraction of genes upregulated during nodule development in *Medicago truncatula* are co-regulated and physically grouped in genomic clusters termed symbiotic islands[1, 2] Symbiotic islands display a distinct epigenetic signature that is dramatically remodeled during the root-to-nodule transition[2] . Yet, the molecular mechanisms enabling these transcriptional and epigenetic reprogramming remain to be uncovered. Remarkably, symbiotic islands are enriched in long non-coding RNAs (lncRNAs), which are emerging regulators of gene expression through interactions with transcription factors and epigenetic regulators[3, 4]. We identified two lncRNAs with nodule-specific expression that we named *SYMBIOTIC NON-CODING RNAs* (*SYNCs*). *SYNCs* are transcribed from two distinct symbiotic islands containing the symbiotic genes *RSD*[5] and *EDN3 (P. Gamas, personal communication)*. *RSD*, *EDN3*, and adjacent genes within their symbiotic islands undergo a dramatic epigenetic reprogramming that is required to unlock their expression in nodules. We hypothesize that *SYNCs* act as regulators of this local transcriptional and epigenetic reprogramming at *RSD* and *EDN3* symbiotic islands to ensure nodule development. By harnessing the well-established *Medicago* - rhizobia symbiotic system combined to genetics, transcriptomics, proteomics, chromatin-based and cell biology strategies, we investigate the epigenetic and transcriptional control of *RSD*, *EDN3*, and their respective symbiotic islands by *SYNCs.* Progress in functional analysis of *SYNCs* using knock-down, knock-out, and overexpression strategies, coupled to expression studies will be presented.

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ORAL PRESENTATION

O 24

THE ROLE OF RPG DURING INFECTION THREAD FORMATION AND PROGRESSION

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Host-controlled intracellular accommodation of nitrogen-fixing bacteria is essential for the establishment of a functional Root Nodule Symbiosis (RNS). In many host plants, this occurs via transcellular tubular structures (infection threads - ITs) that extend across cell layers via polar tip-growth. Comparative phylogenomic studies have identified *RPG* (*RHIZOBIUM-DIRECTED POLAR GROWTH*) among the critical genetic determinants for bacterial infection[1,2]. In *Medicago truncatula*, *RPG* is required for effective IT progression within root hairs[3] but the cellular and molecular function of the encoded protein remains elusive. Here, we show that RPG coalesces with the core endosymbiotic components VAPYRIN (VPY) and LUMPY INFECTION (LIN)[4,5] in infectosome foci and is indispensable for VPY recruitment into these cytoplasmic structures. Fluorescence Lifetime Imaging Microscopy (FLIM) of phosphoinositide species during bacterial infection revealed that functional RPG is required to sustain strong membrane polarization at the advancing tip of the IT. In addition, loss of RPG functionality alters the cytoskeleton-mediated connectivity between the IT tip and the nucleus and affects polar secretion of the cell wall modifying enzyme NODULE PECTATE LYASE (NPL). Our results integrate RPG into a core host machinery required to support symbiont accommodation, suggesting that its occurrence in plant host genomes is essential to co-opt a multimeric protein module committed to endosymbiosis to sustain IT-mediated bacterial infection.

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ORAL PRESENTATION

O 25

CHARACTERIZATION OF THE METABOLIC REGULON OF THE SIBLING NON-CODING RNAs AbcR1 AND AbcR2 IN *Sinorhizobium meliloti*

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Genetic and metabolic reprogramming underwent by rhizobia during symbiosis with legumes has been almost exclusively studied from the perspective of the transcriptional regulation assisted by proteins. However, post-transcriptional regulation by small RNAs (sRNAs) is expected to play major yet unexplored roles in symbiosis[1]. sRNAs of the *trans*-acting class typically target the translation initiation region of multiple mRNAs by protein-assisted short and discontinuous antisense interactions, thereby affecting translation and/or stability of the message. Here, we used a protocol based on MS2-affinity purification coupled with RNA sequencing (MAPS) to decipher the post-transcriptional regulon of the sibling *trans*-sRNAs AbcR1 and AbcR2 previously identified in the alfalfa symbiont *Sinorhizobium meliloti*[2]. This approach unveiled exceptionally large and overlapping AbcR1/2 mRNA interactomes (~6% of the *S. meliloti* genes). Most mRNAs encode transport/metabolic proteins whose translation is silenced by modifiable base-pairing to two distinct anti-Shine Dalgarno motifs identifiable in both sRNAs. A metabolic model-aided analysis of the targetomes predicts changes in AbcR1/2 expression in response to shifts in carbon/nitrogen sources. A relevant number of target mRNAs are differentially expressed in rhizosphere-related conditions. Remarkably, lack of AbcR1 specifically compromised the ability of *S. meliloti* to colonize the root rhizoplane. Thus, AbcR1 likely enables the hierarchical utilization of available substrates and optimizes *S. meliloti* metabolism for the competitive colonization of nutritionally complex environments. Because riboregulation relies on modifiable base-pairing interactions, our findings open new possibilities for engineering the legumes rhizobiome in the sustainable agricultural practices.

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ORAL PRESENTATION

O 26

PTS^{NTR} INTERACTS WITH RELA TO SIGNAL THE INTRACELLULAR NITROGEN AND CARBON BALANCE IN *RHIZOBIUM LEGUMINOSARUM*

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Bacteria constantly adapt to fluctuations in their environment to survive. Optimal resource allocation upon nutritional starvation requires efficient control of metabolism to adjust growth by modulating gene expression. The Nitrogen Phospho-Transferase System (PTS^{Ntr}) in *R. leguminosarum* (PTS^{Ntr}) balances carbon and nitrogen metabolism with a switch utilising the output proteins PtsN and ManX by protein-protein interactions[1], while the master regulator RelA synthesises the alarmone ppGpp(p) in response to stress such as amino acid starvation[2]. We show in this work that the stringent response modulates cell physiology at the transcriptional level in *R. leguminosarum* by sensing the carbon and nitrogen status via interaction with PTS^{Ntr}. The multidomain bifunctional (p)ppGpp synthetase/hydrolase RelA interacts with phoshorylated PtsN to enable activation of RelA under nitrogen starvation. Suppressors of a transposon mutant of PtsP, the first component of PTS^{Ntr}, mapping to the different domains of RelA, had increased amino acid transport, EPS production and metabolic activity, confirming that *relA* secondary mutations were gain of function mutants. This global regulator has the same cellular targets as PTS^{Ntr} and controls genes associated with essential processes and stress adaptation, orchestrating cellular adaptation by transcriptional regulation. Furthermore, RelA is also required for an efficient symbiosis between rhizobia and legumes. The interaction between these regulatory pathways is highly conserved, highlighting the importance of these connections in different bacterial species and in host-microbe interactions.

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ORAL PRESENTATION

O 27

THE AUTOREGULATION OF NODULATION HAS ORIGINATED FROM A MECHANISM THAT RESTRICTS LATERAL ROOT FORMATION

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Serving as atmospheric nitrogen-fixing hubs, symbiotic nodules develop as lateral organs on the majority of legume and some non-legume roots. A homeostatic mechanism, called autoregulation of nodulation (AON), systemically limits their development. In *Lotus japonicus*, AON is mediated by shoot-localized HYPERNODULATED ABERRANT ROOT FORMATIOM (HAR1) receptor kinase which is also present in roots. Deleterious mutations at the *HAR1* locus lead to excessive nodule and lateral root formation, indicative of a role for the receptor in both symbiotic and non-symbiotic root development. The functioning of HAR1 during symbiosis has been well characterized but the mechanism by which this receptor limits lateral root formation and how this relates to AON remains mostly elusive. We have identified the *NUCLEAR FACTOR-Y SUBUNIT A4* (*NF-YA4*) gene as the main target of *HAR1*-dependent regulation in the context of root architecture. In the absence of functional *HAR1*, upregulated expression of *NF-YA4* directs transcriptional reprograming, which accounts for circa 80% of the *har1* mutant-dependent transcriptome. This includes enhanced expression of *LATERAL ORGAN BOUNDARIES DOMAIN (LOB)*, *STYLISH* (*STY)* and *YUCCA*-type *FLAVIN MONOOXYGENASE* genes, which promotes excessive auxin signalling and lateral root emergence. Conversely, removing *NF-YA4* from the *har1-1* background restores wild-type-like root architecture. We show that expression of *NF-YA4* is upregulated in *L. japonicus* roots by auxin and downregulated by low nitrate availability. *NF-YA1,* which in *L. japonicus* regulates nodule emergence at the post-initial cell division stage*,* is the closest paralogue of *NF-YA4*, suggesting that duplication and neofunctionalization of an ancestral *NF-YA* involved in lateral root development has led to its recruitment to the nodule emergence program. Our results indicate that AON evolved from a mechanism that restricts lateral root formation under low nitrogen conditions and that the *NF-YA1*-dependent pathway is one of its targets.

ORAL PRESENTATION

O 28

TRANSCRIPTIONAL REGULATION OF AUTOREGULATION OF NODULATION AT THE SINGLE-CELL LEVEL IN *LOTUS JAPONICUS*

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To limit the number of nodule organogenesis and infection events, legumes have developed a systemic negative feedback loop named autoregulation of nodulation (AON). Upon nodule organogenesis in *Lotus japonicus*, CLAVATA (CLV)/EMBRYO SURROUNDING REGION (ESR)-RELATED PROTEIN (CLE) CLE-RS1/2 peptides are synthesized in the root and are subsequently transported to the shoot. They are perceived by the receptor kinase HYPERNODULATION ABERRANT ROOT FORMATION 1 (HAR1), causing a downregulation of the shoot-to-root mobile *miR2111* in the phloem, which is otherwise transported to the root to trigger mRNA cleavage of the Kelch repeat-containing F-box protein TOO MUCH LOVE (TML). While this general mechanism has been identified, it is still elusive if other tissues in the shoot are responsive to rhizobial infection of the root and whether those participate in the downregulation of miR2111. Moreover, little is known which downstream components ultimately link HAR1 and miR2111. To investigate this, we performed protoplast-based single-cell RNA-sequencing (scRNA-seq) on wild-type and *har1* Gifu shoots two days post water or R7A treatment. All known shoot tissues responded to varying degrees to rhizobial infection, phloem cells were particularly responsive in wild type, while *har1* phloem cells displayed a dampened transcriptional response. We therefore think that our scRNA-seq data set provides the foundation to broaden the mechanistic understanding of AON in *L. japonicus*.

ORAL PRESENTATION

O 29

THE HIDDEN BIODIVERSITY OF *CASUARINA*-INFECTIVE *FRANKIA*

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Actinorhizal root nodule symbioses are entered between a diverse group of mostly woody dicotyledonous plants and nitrogen-fixing soil actinobacteria of the genus *Frankia.* Some of the most ecologically relevant actinorhizal plants are *(Allo-)Casuarina* species, used in shelter belts and phytoremediation in warmer climates. They show high tolerance to different abiotic stresses and thanks to their symbioses can grow on marginal soil. However, while *(Allo-)Casuarina*- infective *Frankia* strains can have, e.g., high salt tolerance *ex planta,* this usually does not transfer to symbiosis. All *Frankia* strains isolated from *(Allo-)Casuarina* nodules growing on four continents belong to a single species, *Frankia casuarinae.* This lack of diversity is unusual for *Frankia;* and there are several examples of the isolation of strains from ostensibly surface-sterilized nodules which could not nodulate the plant the nodules came from. We assessed the situation by direct sequencing of nodule DNA from three countries, removal of plant sequences and assembly of metagenome assembled genomes. It turned out that the dominant strains in field nodules were far more diverse and showed genome reduction compared to *F. casuarinae;* versions of *F. casuarinae* with up to 30% genome reduction were identified. The genome reduction suggests uncultivability. We conclude that the true biodiversity of *(Allo-)Casuarina*-infective *Frankia* strains has not been assessed yet, and that in order to obtain optimized inocula for different abiotic stress conditions, e.g., for salt stress in shelter belts to protect from tsunamis, it is necessary to work with uncultivable strains from crushed nodules.

ORAL PRESENTATION

O 30

INTERACTION OF PHYTOHORMONE SIGNALING PATHWAYS REGULATING ROOT COLONIZATION BY ARBUSCULAR MYCORRHIZA FUNGI

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Arbuscular mycorrhiza is an ancient and wide-spread symbiosis between most land plants and fungi of the glomeromycotina. The symbiosis is based on the exchange of nutrients: the fungi provide mineral nutrients to the plant and receive photosynthetically fixed organic carbon in the form of lipids and carbohydrates in return. The colonization of plant roots by AM fungi is a step-wise process, which is largely under plant control. Phytohormones play a key role in regulating the establishment and maintenance of AM. Among them, the gaseous hormone ethylene has been shown to suppress root colonization by AM fungi, but the underlying molecular mechanisms remained unclear. We found that ethylene signaling modulates the expression of genes required for root colonization by targeting an important player in the karrikin signaling pathway.

ORAL PRESENTATION

O 31

IMPACT OF COMMON SYMBIOSIS SIGNALLING PATHWAY GENES ONTO BARLEY ROOT COLONISATION BY DIAZOTROPHIC BACTERIA

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The Common Symbiosis Signalling Pathway (CSSP) is an essential genetic network required for successful facilitation of symbiosis between plants and arbuscular mycorrhiza fungi, as well as for a functional nitrogen-fixation symbiosis between rhizobia and leguminous plants[1]. In cereals, CSSP genes have shown to have a variety of functions during AM symbiosis, including the induction of signalling cascades, TF regulation and control of strigolactone biosynthesis[1,2,3]. However, studies on the impact of the CSSP onto potential interactions between non-leguminous plants and nitrogen-fixing (diazotrophic) bacteria are limited. A detailed assessment of CSSP gene functions in this context offers the exploration of potential targets for future engineering purposes, battling today's environmental challenge of N-limitation and the necessity for organic fertiliser development. In this study, we used a set of barley CSSP mutants and investigated the impact of the CSSP onto root microbiota structure in soil and synthetic community assays. A competition assay applying >400 diazotrophic bacteria on barley allowed the identification of a set of competitive colonisers, providing useful key candidates for future studies to obtain a better understanding of essential diazotroph characteristics for successful colonisation on cereals, as well as a base for future bacterial engineering purposes to improve nitrogen accumulation. CSSP mutations had significant effects on colonisation of key diazotrophic colonisers, indicating a function of the CSSP in diazotroph recruitment at the root-soil interface. Single inoculation assays supported this hypothesis and root exudate studies gave an insight into potential gene functions and metabolic components involved in the interaction between non-leguminous plants and diazotrophic bacteria.

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ORAL PRESENTATION

O 32

INTERACTION BETWEEN ARBUSCULAR MYCORRHIZAL FUNGI AND RHIZOBIA DURING THE TRIPARTITE SYMBIOSIS WITH *LOTUS JAPONICUS*

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Legume plants establish symbioses with both nitrogen-fixing rhizobia and arbuscular mycorrhizal fungi (AMF). Simulating the natural conditions, where rhizobia and AMF co-exist in soils, we perform double inoculations to study the microbial interactions during the establishment of the legume-rhizobia-AMF tripartite symbiosis. Using the well-studied model legume *Lotus japonicus* and performing inoculations with its microsymbiont *Mesorhizobium loti* and different AMF strains, we observed that rhizobia and AMF do affect one another during the colonization of the same host root. We identified that nodulation is positively affected by AMF, whereas the impact of rhizobia on mycorrhizal colonization is AMF strain dependent [1]. To determine the symbiotic stages that rhizobium and AMF interact, we performed single and double inoculations of *L. japonicus* roots with *M. loti* and AMF at different time points and observed the symbiotic phenotypes testing different inoculation strategies. Moreover, to gain insight into the rhizobium-AMF interaction and identify molecular components that influence the symbiotic outcome in rhizobium-AMF double-inoculated plants, we examined the expression of specific genes. The present study aims to identify main factors that influence the tripartite association of legumes with AMF and rhizobia, in order to make a more efficient use of legume plants in agroecosystems.

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ORAL PRESENTATION

O 33

THE ENDOSYMBIONT OF *EPITHEMIA CLEMENTINA* IS SPECIALIZED FOR NITROGEN FIXATION WITHIN A PHOTOSYNTHETIC EUKARYOTE

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Epithemia spp. diatoms contain obligate, nitrogen-fixing endosymbionts, or “diazoplasts”, derived from cyanobacteria. These algae are a rare example of photosynthetic eukaryotes that have successfully coupled oxygenic photosynthesis with oxygen-sensitive nitrogenase activity. Here, we report a newly-isolated species, *E. clementina*, as a model to investigate endosymbiotic acquisition of nitrogen fixation. To detect the metabolic changes associated with endosymbiotic specialization, we compared nitrogen fixation, associated carbon and nitrogen metabolism, and their regulatory pathways in the *Epithemia* diazoplast with its close, free-living cyanobacterial relative, *Crocosphaera subtropica*. Unlike *C. subtropica*, we show that nitrogenase activity in the diazoplast is concurrent with, and even dependent on, host photosynthesis and no longer associated with cyanobacterial glycogen storage suggesting carbohydrates are imported from the host diatom. Carbohydrate catabolism in the diazoplast indicates that the oxidative pentose pathway and oxidative phosphorylation, in concert, generates reducing equivalents and ATP and consumes oxygen to support nitrogenase activity. In contrast to expanded nitrogenase activity, the diazoplast has diminished ability to utilize alternative nitrogen sources. Upon ammonium repletion, negative feedback regulation of nitrogen fixation was conserved, however ammonia assimilation showed paradoxical responses in the diazoplast compared with *C. subtropica*. The altered nitrogen regulation likely favors nitrogen transfer to the host. Our results suggest that the diazoplast is specialized for endosymbiotic nitrogen fixation. Altogether, we establish a new model for studying endosymbiosis, perform the first functional characterization of this diazotroph endosymbiosis, and identify metabolic adaptations for endosymbiotic acquisition of a critical biological function.

ORAL PRESENTATION

O 34

DEFENSE RESPONSES OF RICE ROOTS ARE DYNAMICALLY SUPPRESSED DURING THE ESTABLISHMENT OF BACTERIAL ENDOPHYTES

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Bacterial endophytes are important contributors to plant health, growth, and nutrient uptake, however, a systematic understanding of how plants establish this specific interaction is still lacking[1]. Transcriptome microarray studies indicated that responses of rice roots to the diazotrophic model endophyte *Azoarcus olearius* BH72 and the pathogen *Xanthomonas oryzae* were drastically different[2]. Using this diazotroph, we employed RNA-Seq to study the timing of molecular events which govern the establishment of endophytic bacteria inside host tissue, including boosted colonization to test the boundaries of defense or beneficial interaction. After an initial induction of pathogen-related genes, a 57-fold increase of endophytic root colonization (1 to 7 DPI) by *Azoarcus* was concomitant with the release of defense responses: Expression of genes for PR-proteins, chitinases, glucanases, and ROS-detoxifying proteins was suppressed, without the involvement of bacterial catalase *katG*. Even boosted colonization did not increase but suppress plant stress. Re-programming included genes for distinct signaling pathways, as well as up-regulation of ammonium transport and assimilation genes. Brassinosteroid signaling is apparently not involved in governing endophyte establishment, as indicated by colonization patterns of rice *OsGSK2* or *OsBZR1* overexpressor mutants. Bacteria appeared to short-circuit the initial root defense responses for a compatible interaction during endophytic establishment, involving previously unsuspected putative rice candidate genes.

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ORAL PRESENTATION

O 35

CO-INOCULATION USING SPECIES OF BRADYRHIZOBIUM AND AZOSPIRILLUM BRASILENSE – THE BRAZILIAN CASE OF SUCCESS ON BIOLOGICAL NITROGEN FIXATION

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Microbial inoculants are biotechnological products used for different purposes, the main one being to replace chemical fertilizers totally or partially, with an emphasis on N-fertilizers, reducing costs and decreasing the contamination of the soil, water, and atmosphere. Brazil has a long tradition in the use of rhizobia inoculants, especially for the soybean crop. In 2009 the first commercial inoculant carrying the plant-growth-promoting Azospirillum brasilense strains Ab-V5 and Ab-V6 reached the market. One decade after the release of these two strains, 17 million doses were commercialized for grasses, including corn, wheat, rice, and pastures, and co-inoculation (the association of two or more species of inoculants) of legumes, such as soybean and common bean. Several research in Brazil presented consistent results of increases in root growth, biomass production, grain yield, uptake of nutrients due to the inoculation with Ab-V5 and Ab-V6. In the soybean season 2021/22 in Brazil, the inoculation rate with Bradyrhizobium to promote biological nitrogen fixation (BNF) in soybean was of 82%, which represents around 33 million of hectares treated. The need of a continuous increase in yield to attend the soybean demand, stimulate farmers to adopt inputs to guarantee the appropriated supply of Nitrogen. Considering that soybean has around 65 kg of N per metric tonne of grain, it is very important to adopt tools that allow greater efficiency of the BNF. The most widely studied is the association of Bradyrhizobium with Azospirillum brasilense. In addition to be a N fixing bacterium, Azospirillum produces hormones, such as auxins, which stimulate the initial root hairs formation. This effect allows the anticipation in the formation of nodules and a greater number of nodules. All these benefits have been observed in many trials in Brazil. Those results have been impacting on the adoption of the co-inoculation in soybeans. According to a Brazilian market research done by SPARK - ANPII, the co-inoculation technique was used in 11.75 million hectares in the soybean season 2021/2022, which represents an adoption rate of 29%, contributing to the sustainable protein production.

Keywords: Co-inoculation, Nitrogen; Azospirillum; BNF.

ORAL PRESENTATION

O 36

BIOLOGICAL NITROGEN FIXATION ON THE AERIAL ROOTS OF MAIZE AND SORGHUM FOR SUSTAINABLE AGRICULTURE

Rafael E. Venado[1], Saptarshi Pyne[2], Jennifer Wilker[1], Vania Pankievicz[1], Paulo Fernandes[1,3], Fletcher Robbins[1], Emily Wolf[4], Saddie Vela[4], Daniel Laspisa[5], Wendell J. Pereira[6], Christopher Dervinis[6], Jason Wallace[5], Wilfred Vermerris[4], Matias Kirst[6], Sushmita Roy[2], and Jean-Michel Ané[1]

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Some maize (*Zea mays*) landraces obtain significant amounts of nitrogen from the atmosphere by hosting diazotrophic communities in the mucilage produced by their aerial roots after rain[1,2]. We identified sorghum (*Sorghum bicolor*) accessions with many aerial roots, producing significant amounts of mucilage. Like the maize mucilage, the sorghum mucilage is a proper environment for several diazotrophs to fix nitrogen. In both maize and sorghum, aerial root development is regulated by soil nitrogen availability and ambient humidity. Using both GWAS and QTL approaches, we have started to identify loci associated with aerial root development and mucilage production. Rain is required to trigger mucilage production by the root cap and border cells present on aerial roots. Aerial roots of maize and sorghum produce exceptionally abundant and large border cells[3]. We used bulk and single-cell RNA-seq approaches to better understand border cell development and mucilage production. At the same time, we isolated over 200 diazotrophic bacteria from maize and sorghum mucilage. Using small synthetic communities, we demonstrated that some communities promote nitrogen fixation. Progress toward breeding for the trait in maize and sorghum will be reported.

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ORAL PRESENTATION

O 37

LIGHT SENSITIVE SHORT HYPOCOTYL (LSH) GENES CONFER SYMBIOTIC NODULE IDENTITY IN *MEDICAGO TRUNCATULA*

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Legumes grow specialized root nodules to host beneficial nitrogen-fixing bacteria that provide the plant with ammonia in exchange for carbon. These symbiotic nodules are distinct from lateral roots in morphology and function as they comprise of cells that can accommodate nitrogen-fixing rhizobial bacteria intracellularly and provide favorable conditions for the biological nitrogen fixation process. Nodules initiate from the inner tissue layers in response to the perception of rhizobial bacteria at the root surface via cytokinin-mediated upregulation of the nodulation-specific transcriptional regulator *NODULE INCEPTION (NIN)*. Our previous findings that the initiation of lateral roots and nodules converges at a common developmental program[1] led to the hypothesis that an additional nodule-specific program is required to determine nodule organ identity on top of the shared root-like initiation program. Here, we show that two members of the *LIGHT SENSITIVE SHORT HYPOCOTYL (LSH)* transcription factor family (*MtLSH1* and *MtLSH2*), predominantly known to define organ boundaries and meristem complexity in the shoot, function as regulators of nodule organ identity. *MtLSH1*/2 are upregulated during early stages of nodule development in a cytokinin- and *NIN*-dependent manner and are expressed in dividing cells. Our loss of function analysis of *lsh1/2* demonstrated that these regulators are required for the development of functional nodule primordia that can support the intercellular cortical infection, the intracellular colonization, and nitrogen-fixation by the bacteria. Furthermore, molecular functional analysis revealed that *LSH1/2* control components of the auxin-cytokinin cross talk and function upstream of and together with the previously identified nodule organ identity genes nuclear factor *Y-A1* (*NF-YA1)* and *NODULE ROOT1/2 (NOOT1/2)* to recruit a program with pleiotropic functions in the shoot to differentiate nodules from lateral roots and to determine nodule organ identity. The principal outcome of *LSH1/LSH2* function is the production of cells able to accommodate nitrogen-fixing bacteria, the unique nodule feature. We conclude that the coordinate recruitment of a pre-existing primordium identity program, in parallel to a root initiation program, underpins the divergence between lateral roots and nodules.

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ORAL PRESENTATION

O 38

LONG NON-CODING RNAS MAY FACILITATE THE REGULATORY ACTIVITY OF THE PIONEER TRANSCRIPTION FACTOR NFYA1 DURING NODULE DEVELOPMENT

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The transcription factor NF-YA1 regulates nodule development during the legume-Rhizobia symbiosis [1,2,3,4]. NF-Y transcription factors that bind CCAAT boxes in promoters have in addition been shown to act as pioneer transcription factors in both animals and plants [5,6]. We have started to accumulate evidence that NF-YA1 also acts as a pioneer transcription factor in *Medicago truncatula* regulating the root to nodule transition. In recent years, long non-coding RNAs (lncRNAs) have been described as a new class of regulators with roles in a wide range of cellular regulatory mechanisms, including the control of gene expression *via* chromatin modifications [7]. Comparative transcriptomics have revealed several lncRNAs activated during early steps of the legume-rhizobium symbiosis, and under the control of NF-YA1. In addition, using a RNA-immunoprecipitation (RIP) strategy, we have identified two lncRNAs physically interacting with NF-YA1. Hence, lncRNA-interacting with or regulated by NF-YA1 may help to regulate gene expression and consolidate the pioneering activity of these transcription factors in the context of nodule development. Progress in functional and expression studies of these lncRNAs during the *Medicago* - Rhizobia symbiosis will be presented.

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ORAL PRESENTATION

O 39

THE MEDICAGO SYMCEP7 HORMONE PROMOTES NODULATION FROM SHOOTS WITHOUT PENALIZING LATERAL ROOT NUMBER

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Root nodule and lateral root (LR) formation are energetically costly. How legumes simultaneously balance the cost of supporting nodule and/or LR formation to acquire soil nutrients is poorly understood. Under low N, Medicago negatively controls nodule number by the systemic autoregulation-of-nodulation (AON) pathway via a sub-class of CLAVATA 3-RELATED (CLE) peptide hormones[1]. Conversely, specific members of Medicago C-TERMINALLY ENCODED PEPTIDE (CEP) hormone family, which produce mature 15 amino acid secreted peptides, boost nodule number by counteracting the AON pathway and suppressing the effects of ethylene[2,3]. Most *CEP* family members, however, negatively control LR number without promoting nodulation[4]. Here, we used mass spectrometry to demonstrate that Medicago *CEP7* produces a CEP hormone variant, SymCEP7, with a symbiosis associated activity that does not affect LR number. Rhizobial inoculation and Nod factors rapidly induce *CEP7* expression via NIN[5] and, unlike other CEP genes tested, *CEP7* is hyper-expressed in EIN2 mutants[6]. Using fluorescence microscopy and expression analysis, we showed that SymCEP7 activity requires the CRA2 CEP receptor and activates the shoot-to-root systemic effector, miR2111[5,7]. Shoot applied SymCEP7 promotes nodulation in the pM to nM range, whereas concentrations up to five orders of magnitude higher were needed by root applied SymCEP7 to mediate similar effects. Shoot applied SymCEP7 also promoted nodule number in White Clover and Lotus, which suggests that its biological function may be evolutionarily conserved. We propose that SymCEP7 has a symbiosis-associated activity and acts in the Medicago shoot to counterbalance the AON pathways to enable nodulation without compromising lateral root growth, thus promoting the acquisition of a full range of nutrients to support growth under suboptimal N conditions.

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ORAL PRESENTATION

O 40

SINGLE-CELL ANALYSIS OF LINEAGES TRANSITIONS DURING NODULE DEVELOPMENT IN *Medicago truncatula*

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Nitrogen fertilizer production relies heavily on natural gas, contributing to climate change. Understanding root nodule symbiosis (RNS) in legumes like *Medicago truncatula* can help engineer RNS in non-nodulating plants, reducing fertilizer dependence. Single-nuclei RNA sequencing (snRNA-seq) has been used to identify genes involved in the control of many biological processes, specifying their expression patterns at the cell type level. Applying this technology for RNS allows the characterization of all genes in an unprecedented resolution, together with the discovery of new candidate regulators of RNS. In this study, single-nuclei RNA sequencing (snRNA-seq) was used to explore the transcriptional response of *M. truncatula* roots to *Sinorhizobium meliloti* infection. We sampled two genotypes, Jemalong A17, and the hypernodulation mutant *sunn-4*, at four-time points: control (0h), 24h, 48h, and 96h post-infection. Transcriptional changes were characterized for root hair infection and nodule development in response to *S. meliloti*. Pseudotime analysis reconstructed the developmental trajectories of those processes by ordering the cells according to their transcriptional states and identifying genes that potentially govern them. The data revealed that, as the response to the infection progresses, different members of gene families involved in RNS exhibit distinct expressions according to the cell types, providing insights into the genes co-opted for RNS. For example, the knockdown of a member of the STYLISH family, specifically expressed in cells responding to the infection, confirmed its involvement in RNS. Ongoing experimental validation of other candidate genes may uncover new factors governing the transcriptional changes necessary for RNS establishment in non-nodulating crops.

ORAL PRESENTATION

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A HIGH-RESOLUTION TRANSCRIPTOMIC ATLAS DEPICTING NITROGEN FIXATION AND NODULE DEVELOPMENT IN SOYBEAN

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Although root nodules are essential for biological nitrogen fixation in legumes, the cell types and molecular regulatory mechanisms contributing to nodule development and nitrogen fixation in determinate nodule legumes, such as soybean (*Glycine max*), remain incompletely understood. Here, we generated a single-nucleus resolution transcriptomic atlas of soybean roots and nodules and annotated 17 major cell types, including six that are specific to nodules. We identified the specific cell types responsible for each step in the ureides synthesis pathway, which enables spatial compartmentalization of biochemical reactions during soybean nitrogen fixation. By utilizing RNA velocity analysis, we reconstructed the differentiation dynamics of soybean nodules, which is differed from those of indeterminate nodules in *Medicago truncatula*. Moreover, we identified several putative drivers of soybean nodulation and two of these genes, *GmbHLH93* and *GmSCL1*, were as-of-yet uncharacterized in soybean. Overexpression of each gene in soybean hairy root systems validated their respective roles in nodulation. Notably, enrichment for cytokinin-related genes in soybean nodules led to identification of the cytokinin receptor, *GmCRE1*, as a prominent driver of nodulation. *GmCRE1* knockout in soybean resulted in a striking nodule phenotype with decreased nitrogen fixation zone and markedly fewer symbionts, accompanied by downregulation of nodule-specific gene expression, as well as almost complete abrogation of biological nitrogen fixation. In summary, this study provides a comprehensive perspective of the cellular landscape during soybean nodulation, shedding light on the underlying metabolic and developmental mechanisms of soybean nodule formation.

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STRIGOLACTONES REPRESS NODULE DEVELOPMENT AND SENESCENCE IN PEA

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Strigolactones are a class of phytohormones that are involved in many different plant developmental processes, including the rhizobium-legume nodule symbiosis. Although both positive and negative effects of strigolactones on the number of nodules have been reported, the influence of strigolactones on nodule development is still unknown. Here, by means of the *ramosus* (*rms*) mutants of *Pisum sativum* (pea) cv Terese, we investigated the impact of strigolactone biosynthesis (*rms1* and *rms5*) and signaling (*rms3* and *rms4*) mutants on nodule growth. The *rms* mutants had more red, i.e., functional, and larger nodules than the wild-type plants. Additionally, the increased nitrogen fixation and senescence zones with consequently reduced meristematic and infection zones indicated that the *rms* nodules developed faster than the wild-type nodules. An enhanced expression of the nodule zone-specific molecular markers for meristem activity and senescence supported the enlarged, fast maturing nodules. Interestingly, the master nodulation regulator, *NODULE INCEPTION, NIN*, was strongly induced in nodules of all *rms* mutants but not prior to inoculation. Determination of sugar levels with both bulk and spatial metabolomics in *rms* roots and nodules, respectively, hints at slightly increased malic acid levels early during nodule primordia formation and reduced sugar levels at later stages, possibly the consequence of an increased carbon usage of the enlarged nodules, contributing to the enhanced senescence. Taken together, these results suggest that strigolactones balance the development of nodules which is probably mediated through NIN, and with the available plant sugars.

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A NOVEL MOTIF OF NIN DETERMINES ITS DIFFERENT DNA-BINDING SPECIFICITY FROM NLPs

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In the root nodule symbiosis (RNS) of leguminous plants, NODULE INCEPTION (NIN) is an essential transcription factor (TF) regulating expression of many nodulation genes. NIN is thought to be derived from NIN-LIKE PROTEINs (NLPs), which are responsible for nitrate response in plants, in the RNS clades[1]. In *Lotus japonicus*, we have recently shown that LjNIN and LjNLP4 have clearly different DNA-binding property despite the high similarity of their RWP-RK DNA-binding domains and binding cis-elements[2]. This difference in DNA-binding specificity allows only NIN to induce the expression of nodulation genes. However, it remains elusive how these similar TFs have different DNA-binding specificity.

Here, we identified a novel amino acid motif, which we named NIN-specific motif (NSM). NSM is conserved among NINs of nodulating plants but does not belong to any known domains. We showed that NSM is involved in the regulation of NIN-specific target genes and responsible for the difference in DNA-binding specificity between LjNIN and LjNLP4. Of note, a modified LjNLP4 with NSM could regulate the expression of a NIN-specific target gene. Therefore, the incident that NIN has evolutionally obtained NSM may be a significant event to establish RNS.

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DOES AUXIN ACT AS A SWITCH FOR NIN ACTIVATION DURING ACTINORHIZAL NODULE DEVELOPMENT?

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Biological nitrogen fixation requires *de novo* organogenesis of root nodules where nitrogen fixing bacteria fix atmospheric nitrogen into ammonia. A monophyletic clade of four angiosperm families namely FaFaCuRo can undertake this symbiotic programme with either filamentous actinorhizal bacteria or with rhizobia in a mutually exclusive fashion. The transcription factor (TF) NIN (Nodule Inception) belonging to the NLP (NIN-Like Protein) superfamily plays the most important role in this organogenesis process. While NLPs have recently been reported for their N-sensing capability and role in lateral root organogenesis[1], it is apparent that the nitrogen fixers neo-functionalised one of the NLPs (NIN) to act as a bridge between putative NLP functions and bacterial signal perception to initiate a nodule development programme. Research in model Fabaceae indicates that NIN activates the lateral root development pathway and deploys auxin signalling to induce cell division[2]. While NIN induces auxin biosynthesis in legumes, it is in turn activated by cytokinin responsive elements in its promoter[3]. Our study of newly sequenced genomes of 15 actinorhizal nodulating species shows that this cytokinin element is missing in their NIN promoters. On the other hand, we could find auxin responsive elements within -2kb of the transcription start site that are also present in all other NLPs, suggesting an ancestral switch. We hypothesise that actinorhizal nodulators, representing a more ancestral form of nodulation, retained the auxin control of NIN. In contrast in the Fabaceae, the addition of cytokinin elements led to differences in NIN activation. Our study of actinorhizal NIN in hairy roots of *Datisca* and *Medicago* shed light on how NIN regulation has evolved during actinorhizal nodulation and how evolution of cytokinin elements added a shift in control of NIN. This work highlights the need for comparative studies to understand the stepwise evolution of nodulation and changes associated with major shifts in the bacterial symbionts.

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ORAL PRESENTATION

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PARASPONIA NODULE ORGANOGENESIS CRACKS THE DOOR FOR RHIZOBIUM

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Parasponia represents an isolated lineage of five nodulating species in the Cannabaceae (order Rosales). It differs from other nodulating non-legume species in that it nodulates with rhizobia rather than *Frankia sp.*. Phylogenomic studies support the hypothesis that the nitrogen-fixing nodulation trait has a single evolutionary origin in the last common ancestor of the orders Rosales, Cucurbitales, Fagales, and Fabales (collectively known as the nitrogen-fixing clade). This hypothesis dictates the occurrence of microsymbiont switches. The most parsimonious scenario is that *Frankia* represents the ancestral microsymbiont, followed by two microsymbiont switches allowing an ancestral legume (Fabaceae, Fabales) and an ancestor of *Parasponia* to accept rhizobia as microsymbiont. Recent adaptations in the HAEMOGLOBIN protein suggest that the switch to rhizobia occurred only recently in *Parasponia*. However, the underlying mechanism that allowed rhizobium to infect *Parasponia* remains elusive. We reconstructed a fate map of *Parasponia* nodule organogenesis. It revealed that the first rhizobium-induced cell divisions occur in the root epidermis, creating novel cracks colonized by the bacteria. We examined allele-specific expression in an F1 hybrid cross between a nodulating *Parasponia* and its non-nodulating sister species *Trema* to reveal *Parasponia*-specific cis-regulatory adaptations in genes associated with this unique crack entry mechanism. Functional analysis of these candidate genes will provide insights into the evolutionary path that allowed a microsymbiont switch in the *Parasponia* lineage.

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ARTIFICIAL EVOLUTION OF ARTIFICIAL PLANT-ASSOCIATED BACTERIAL COMMUNITIES

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Photosynthetic organisms, such as land plants and algae, release organic compounds to the surrounding environment, which create a niche for colonization by heterotrophic microbes. These microbes consume photosynthates and assemble into complex communities, often providing their host with beneficial services in exchange, such as pathogen protection, or enhanced nutrient mobilization. While these interactions are ubiquitous in nature, and of great ecological and agricultural importance, direct evidence for adaptation to their host, and simultaneously to other community members remains scarce. Here, we introduce an experimental evolution experiment using artificial microbial communities derived from roots and nodules of Lotus japonicus and Arabidopsis thaliana. These reductionist experimental systems allow us to study the dynamic behaviour and evolution of host-associated microbes in a community context and provide data that suggest rapid and reproducible bacterial adaptation to their associated photosynthetic host species and to other microbiota members.

ORAL PRESENTATION

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ORM-MEDIATED REGULATION OF SPHINGOLIPID BIOSYNTHESIS IS ESSENTIAL FOR LATERAL ROOT BASE-NODULATION IN *AESCHYNOMENE EVENIA*

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Legumes are able to establish symbiotic interactions with nitrogen-fixing rhizobia that are accomodated in root-derived organs, the nodules. While in most cases, nodules directly form on the root system, some tropical legumes species resort to lateral root-base nodulation. Despite the distinctness of this alternative nodulation process, the underlying molecular mechanisms remain elusive. Here, we show that *Aeschynomene evenia* OROSOMUCOID PROTEIN 1 (AeORM1), a negative regulator of sphingolipid biosynthesis, is required for lateral root-base nodulation of this legume species. Using forward genetics in *A. evenia*, we identified three lines with mutations in *AeORM1* that lead to numerous early aborted nodules, exhibiting defense-like reactions, and shortened lateral roots while the mycorrhizal symbiosis was not altered. *AeORM1* was expressed during lateral root initiation and elongation, including at lateral root bases where nodule primordia form in the presence of symbiotic *Bradyrhizobium*, and then in developped nodules. Mutations in *AeORM1* leaded to sphingolipid overaccumulation in roots and nodules, and lack of reprogramming of sphingolipid composition that normally occur during nodule maturation. Our findings reveal a shared requirement of AeORM1-regulated sphingolipid homeostasis for lateral root development and nodule symbiosis and provide cues on how lateral root base-nodulation is orchestrated.

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EVOLUTION AND DIVERSITY OF *MESORHIZOBIUM* LEGUME SYMBIONTS

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Rhizobia have comparatively large and structurally complex genomes, consisting of a chromosome with the addition of plasmids and/or chromids of varying sizes. The location of symbiosis genes (i.e. *nod*, *nif* and *fix*) within these genomes is genus-specific, with some genera (e.g. *Sinorhizobium* and *Rhizobium*) encoding these genes on plasmids, while others (e.g. *Mesorhizobium* and *Bradyrhizobium*) encode them chromosomally. In *Mesorhizobium* spp., symbiosis genes are chromosomally encoded on genomic islands called symbiosis Integrative and Conjugative Elements (ICEs). Symbiosis ICEs lack their own origin of replication, instead undergoing replication along with the host bacterial chromosome. Symbiosis ICEs can excise from the donor chromosome, transferring to a recipient cell by conjugation, and integrating into the chromosome at specific conserved sequences. ICEs can be monopartite or tripartite in structure and share related ICE transfer and regulation genes, suggesting they evolved from a common ancestral ICE[1,2]. Symbiosis ICE conjugal transfer, integration and excision is stringently regulated so ICEs are not readily lost from host genomes and can be difficult to cure from strains in the laboratory. ICE transfer rates vary, with monopartite ICEs generally transferring at higher rates than tripartite ICEs[1,3]. In the soil, symbiosis ICEs can transfer from inoculum *Mesorhizobium*, resulting in the evolution of new legume microsymbionts. Recipients of these ICEs appear to be non-symbiotic soil *Mesorhizobium* spp., which presumably exist saprophytically as part of the normal microbial community[2]. Some, but not all, of these non-symbiotic *Mesorhizobium* can acquire symbiosis genes *in vitro*. While strains that receive symbiosis ICEs acquire the ability to nodulate legume hosts, they may not fix N_2 efficiently, even though the symbiosis ICE was acquired from a highly effective donor strain[2,3]. This suggests an interaction between the core and symbiosis ICE accessory genome exists in rhizobia, which can significantly influence N_2 fixation efficiency.

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RECONSTRUCTION OF NITROGENASE-OXIDOREDUCTASE FAMILY TREE REVEALS THE EVOLUTIONARY HISTORY OF NITROGEN FIXATION AND ITS EVOLUTIONARY TIMING

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Nitrogen fixation is thought to be an ancient metabolism that developed on early Earth. Although geological records suggest that the metabolism traces back to at least 3.2 billion years ago (Ga)[1], molecular evidence for the origin has yet to be obtained. In this study, we revealed the timing of the origin through reconstruction of the evolutionary history of the protein family that includes proteins involving nitrogen fixation (NifDKEN), comparative genomics, and molecular clock analyses.

Based upon 12,934 nitrogenase-oxidoreductase family proteins scattered across 19,972 prokaryotic genomes and their homology and synteny, the family could be divided into 26 groups, including 15 with no biochemical characterization. Rooting the tree with an enzyme considered to take a primitive form of the family, Ni^{2+}-sirohydrochlorin diamide reductase (CfbD), most groups were divided into two supergroups – each related to nitrogenase subunits NifD and NifK, respectively. Molecular clock analysis of the rooted tree estimated that nitrogen fixation-related proteins began to diversify around 3.0-2.3 Ga, which coincides with geological signatures (3.2-2.75 Ga)[1]. Moreover, the reconstructed evolutionary history provides novel insights into the ancestral form of nitrogen fixation and its chronological and phylogenetic relationship with other proteins in the family, including the placement of nitrogen fixation after the origin of phototrophy.

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ORAL PRESENTATION

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CONVERGENCE AND DIVERGENCE OF NODULE SPECIFIC CYSTEINE RICH PEPTIDES IN DIVERSE LEGUME CLADES

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Legume plants under nitrogen deficiency can enter a symbiotic interaction with N2-fixing soil bacteria called rhizobia. In five legume clades, an exploitive strategy called Terminal Bacteroid Differentiation (TBD) has evolved in which rhizobia undergo extreme differentiation. Terminally differentiated bacteria are larger, polyploid, have a permeabilized membrane, do not replicate, and are better at N2-fixation, providing a higher return on investment for the plant. We know that in several members of the distantly related Inverted Repeat Lacking Clade (IRLC, e.g., Medicago spp.) and the Dalbergioid clade (e.g., Aeschynomene spp.), this differentiation process is triggered by a set of apparently unrelated plant antimicrobial peptides with membrane damaging activity known as Nodule-specific Cysteine-Rich (NCR) peptides. However, whether NCR peptides or similar peptides are also found in other clades where this occurs, and the evolutionary relation among these peptides, remain unknown. Here, to address the molecular identity of NCR peptides and their evolution in different legume clades, we performed inter and intra-clade comparisons of NCR peptides in IRLC and Dalbergioid clades.

First, we collected genomic and proteomic data of species for which NCR peptides are known (4 IRLC and 2 Dalbergioids species, 1750 NCR in total). We then used sequence similarity-based clustering to regroup NCR peptides, resulting in over 400 different NCR homologous groups, each of which was clade specific. We obtained hidden Markov models for each cluster and used the models to predict NCR peptides in plants promoting TBD (7 IRLC, 2 Dalbergioid, 1 Genistoid) or not promoting TBD (1 Robinoid, 4 Milletioid) using whole genome sequences and the Small Peptides Alignment Discovery Application (SPADA) pipeline and transcriptome matching. This resulted in 3300 predicted NCR peptides, of which 30, 12 and 56 were found in the Genistoid, Robinoid, and Milletioid plants, respectively. This result demonstrates that NCR peptides are found in other legume clades that promote TBD.

Next, we obtained high-confidence structural models for one sequence of each of the 400 homologous group and performed structure-based clustering of all models, which resulted in 24 superclusters of IRLC and 2 superclusters of Dalbergioids. i.e., we found no supercluster containing structures from different clades, which is consistent with the idea that NCR peptides evolved convergently in the different clades. Our study further revealed that within each clade NCR evolution is a mix of divergent and convergent processes. Whether the independently evolved NCR are functional analogs in the context of the symbiosis remains to be determined.

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RHIZOBIUM LEGUMINOSARUM POPULATIONS IN MULTI-HOST COMMUNITIES REVEAL DIVISIONS BETWEEN SYMBIOTIC AND FREE LIVING LIFE STYLES

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The rhizobial-legume symbiosis is highly host-specific, thus plant hosts might be expect to exert strong genetic structure on the populaiton, even across small spatial scales. However, as facultative symbionts, rhizobia also spend considerable time as freeliving bacteria in diverse rhizosphere communities. Rhizosphere populations have the potential to play an important role in rhizobial ecology both as a pool of potential symbionts and a hotspot for horizontal gene transfer yet the relationships between nodulating- and free living populations is not well understood. Comparing 120 nodule and rhizosphere Rhizobium leguminosarum isolates from two co-occuring legumes, Common Vetch and White Clover, at multiple sites I show that nodulating and freeliving isolates form distinct genetic populations, with free-living bacteria having greater genetic diversity than those living in the roots.. Rhizosphere isolates also showed greater spatial structure between collection sites with different clades dominant at different locations, while nodule isolates showed far less variability. These data revel divisions between the rhizobia which are actively forming symbiotic associations and those existing freely in the soil. Surprisingly, plant host species exerted relatively little structure on the population, suggesting a role for horizontal gene transfer in keeping symbiosis ‘in the family’ (or genospecies).

ORAL PRESENTATION

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FIXATION THREAD-FORMING BRADYRHIZOBIA ARE GENETICALLY DIVERSE

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The 'symbiosome' (SYM) is the most prevalent anatomical layout of symbiotic nitrogen-fixing bacteria in legume nodules[1]. It initiates upon rhizobial release from the infection thread (IT) when plant cell wall invagination forms a tube (i.e., the IT) by which the rhizobia travel to the nodule primordium. There, they are released into the host cytoplasm where they are only surrounded by a membrane derived from the plant plasmalemma[1]. The lesser studied anatomical layout is the 'fixation thread' (FT), whereby the rhizobia are not released from the IT and instead remain enclosed within a structure consisting of the host cell wall and plasmalemma (i.e., the FT)[1]. Fixation thread nodules are only encountered in the few nodulating non-mimosoid members of the Caesalpinioideae and in some papilionoid genera[1]. Here we studied rhizobial strains isolated from FT-forming hosts, such as *Dimorphandra*, *Erythrophleum*, *Melanoxylon*, *Moldenhawera* and *Chamaecrista*[1]. The isolated strains predominantly belong to the genus *Bradyrhizobium*. Whole genome sequencing enabled phylogenetic analyses of housekeeping as well as symbiosis *nod* and *nif* loci to reveal relationships among all *Bradyrhizobium* type strains as well as representatives of different mobile genetic element types from Weisberg et al.[2]. Although the resulting phylogenies showed some exclusive lineages, most strains from the three nodule 'types' (SYM-forming, FT-forming or both) are genetically diverse across the different loci. Yet, some FT and SYM strains are most closely related to each other. Therefore, current data suggest that FT bradyrhizobia are diverse regarding their core and accessory genomes.

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ORAL PRESENTATION

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UNDERSTANDING NITROGEN-FIXING ROOT NODULE SYMBIOSIS IN PEA (PISUM SATIVUM) POPULATION THROUGH QUANTITATIVE GENETICS

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Plant root nodule symbiosis (RNS) with mutualistic nitrogen-fixing bacteria is restricted to a single clade of angiosperms, the Nitrogen-Fixing Nodulation Clade (NFNC), and is best understood in the legume family. It is widely accepted that nodulation syndrome is quite complex in developmental regulation, and evolutionary genetics, originated through the assembly of modules recruited from existing functions, such as mycorrhizal symbiosis, polar growth, and lateral root development. It is still an open question on whether or not the functional RNS originated once or invented multiple times convergently, the key challenge to understand this question is through a better understanding of the biology and genetics of nodulation. Quantitative genetics offers an under-explored route to identify genes, and regulators of these modules and processes. Taking advantage of the rich natural variation present for RNS characteristics in the century-old classical plant model, the Mendel pea (*Pisum sativum*), we aims to carry out association genetics study, by combining the whole-genome association study (GWAS) from a well-defined global diversity panel with 707 accessions, via the construction of the population genetic variation map and deep phenotyping in root, nodule and many other agronomic traits, with the linkage mapping and heritability estimates of RNS-related traits. We also build a super NAM RILs for high-throughput phenotyping of the nodulation-related traits in the mapping population, such as nodule number, nodule fresh weight, nodule dry weight, root length, root fresh weight, plant height, number of stem nodes, shoot fresh and dry weight. These resources and analyses provide us a platform for nodule-gene discovery and novel nodulation-related alleles mining.

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ORAL PRESENTATION

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A NITROGEN FIXING SYMBIOSIS-SPECIFIC PATHWAY REQUIRED FOR LEGUME FLOWERING

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Flowering time is crucial for plant survival and reproduction and is controlled by flowering pathways [1]. It has long been assumed that fixed nitrogen promotes plant growth and increases reproductive success, but the regulatory mechanism remains unknown. The observations that nodule number and nitrogen fixation peak at when plants flower suggest a strong correlation between Symbiotic nitrogen fixation (SNF) and flowering time, but whether two traits are connected and how SNF and flowering time are coupled remain elusive. Previously we found that miR172c is induced significantly by rhizobia and maintains high level in the nodule[2], and this led us to hypothesize that symbiotic miR172c moves to the shoot to promote flowering. To proof the hypothesis, we conducted a systemic study using combinatorial approach. We show that the symbiotic miR172c moves from nodules/roots to leaves to promote flowering; while fixed nitrogen is transported from the nodules/roots to the leaves to induce miR172c. Thus, symbiotic signal miR172c and nitrogen systemically and synergistically convey symbiotic and nutritional cues from nodules/roots to leaves to accelerate soybean (*Glycine max*) flowering. Furthermore, we found that the combinations of symbiotic miR172c and local miR172c led to activation of florigen-encoding *FLOWERING LOCUS T* (*FT*) homologs (*GmFT2a/5a*) by repressing the flowing repressor*TARGET OF EAT1-like 4a* (*GmTOE4a*). Thus, FTs trigger reproductive development, which allows legumes to survive and reproduce under low-nitrogen conditions. Finally, we showed that this symbiotic specific flowering pathway is conserved in legumes[3]. Together, our discovery has defined a legume specific flowering pathway that integrates symbiotic nodulation and flowering in legumes.

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ORAL PRESENTATION

O 55

FUN: A ZINC REGULATED TRANSCRIPTION FACTOR MEDIATES THE REGULATION OF NITROGEN FIXATION BY THE ENVIRONMENT

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Symbiotic nitrogen fixation provides legume plants with adequate nitrogen; however, it is sensitive to the environment, e.g. nitrogen availability and abiotic stress. To improve nitrogen-fixating efficiency, it is critical to have a thorough understanding of how environmental factors suppress nitrogen fixation. Here, nitrate, the most abundant nitrogen source in nature and strong inhibitor of nitrogen fixation, is used to study the suppression of nitrogen fixation. We found that nitrate suppresses nitrogen fixation via a novel transcription factor-Fixation Under Nitrate (FUN), which is exclusively expressed in nodules. Surprisingly, the micronutrient Zn acts as a secondary signal to regulate the activity of FUN. Zn can force FUN from a dimer (active form) into a filamentous structure (inactive form). Interestingly, Zn is reallocated in nodules after nitrate treatment, which facilitates FUN release from the filamentous form to regulate nitrogen fixation. This demonstrates that Zn can act as a secondary signal to regulate plant development and fitness. There is great potential in transferring this knowledge into legume crops to improve their nitrogen-fixation efficiency, which will benefit sustainable agriculture and the green transition.

ORAL PRESENTATION

O 56

A NITROGEN-INDUCED PEPTIDE MEDIATES NUTRIENT-DEPENDENT REGULATION OF ROOT NODULE SYMBIOSIS

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Plants adjust many nutrient-related physiological processes such as root nodule symbiosis (RNS) by integrating external and internal nitrogen nutrient status. Leguminous plants have a mechanism to control RNS in response to the amount of nitrate in the soil. While the molecular mechanisms controlling RNS by external nitrogen nutrient have been well understood (1, 2, 3), those in response to internal nitrogen nutrient remains mostly elusive.
In this study, we designed a transcriptome analysis to fucus on changes in the internal nitrogen status during nodulation in *Lotus japonicus*. Given that nutritional Information could be mediated by signaling molecules that function systemically between shoot and root, we investigated the expression of genes encoding potentially mobile small proteins. Consequently, a series of genes encoding a small peptide family were found to be upregulated in the shoot by nitrogen fixation products. Analyses of knockout mutants created by CRISPR-Cas9 system indicated that the identified peptides play significant roles in the regulation of RNS through the provision of an essential nutrient to the nodules. These findings uncover a novel nutrient-dependent signaling system required for the establishment of RNS. In addition, we revealed that this peptide family has a conserved role in the regulation of nitrogen homeostasis in plants.

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ORAL PRESENTATION

O 57

NLP2 REGULATION OF *NITRITE REDUCTASE* IS REQUIRED FOR VACUOLE INTEGRITY IN N-FIXING CELLS OF *MEDICAGO TRUNCATULA* UNDER HIGH NITRATE

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NIN-Like Protein 2 (*NLP2*) has very high expression in the N-fixation zone of mature nodules of *Medicago truncatula* where it directly activates the expression of *leghemoglobins* through “double” Nitrate Responsive Elements (dNREs). The *nlp2-1* mutant shows a normal reduction in nodulation and N-fixation in the presence of 5.0 mM nitrate, suggesting that NLP2 does not play a role in nitrate-suppression of nodulation. However, in contrast to WT, nodules that developed on *nlp2-1* under high nitrate frequently exhibited abnormal vacuole morphology, which is not seen at lower nitrate concentrations (0-, 0.5-, and 2-mM nitrate). This was associated with increased numbers of amyloplasts, increased starch content, and higher expression of starch synthesis genes which specifically occurred at 5.0 mM KNO_3. Further investigation revealed increased levels of nitric oxide (NO) and nitrite and decreased Nitrite Reductase (NiR) activity. Transcript profiling revealed that *Nitrite Reductase* expression was strongly reduced in *nlp2* nodules at all nitrate concentrations and that a set of genes that was specifically deregulated in *nlp2* nodules at 5.0 mM KNO_3. This included decreased expression of several genes related to hypoxia, such as *Alcohol Dehydrogenase*, *Pyruvate Decarboxylase (PDC)*, *Phytoglobin3 (Glb3)* and increased expression of *S-Nitrosoglutathione Reductase* (*GSNOR*) which is involved in NO metabolism. This was associated with lower levels of ATP and a higher NAD+/NADH ratio, suggesting that energy metabolism is compromised in *nlp2* nodules at high nitrate. We then tested the role of *NiR* by expressing it in nodules of *nlp2-1* mutants grown at 5.0 mM KNO_3. This rescued the vacuole phenotype and restored the expression of *Glb3-2*, *GSNOR* and *PDC* to normal levels. Overall, our data suggests that NLP2 plays a role in energy maintenance under high nitrate through its regulation of *NiR*.

ORAL PRESENTATION

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THE ROLES OF APOPLASTIC BARRIER IN ROOT NODULE SYMBIOSIS

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In roots, the innermost cortex layer, known as the endodermis, surrounds the central vasculature and contains a diffusion barrier known as the Casparian strip (CS)[1]. This barrier controls the flow of solutes and signals between the environment and the stele by forcing solute exchange to occur over the plasma membrane[1]. In *Arabidopsis thaliana*, CS initiation is governed by a transcription factor MYB36[2] and several associated signalling cascades [3,4,5]. Yet, it remains to be investigated if these systems are conserved in species forming rhizobial symbiosis. Under nitrate starvation conditions, legumes can form root nodules by interacting with rhizobia to fix atmospheric nitrogen[6] Nodule formation is restricted to a narrow developmental window of root, termed the susceptible zone (SZ)[7] Intriguingly, this zone is developmentally adjacent to CS establishment, yet the influence of CS formation on nodule formation is unclear. In this work, we show that although the CS-establishing machinery is conserved in nodulating species, where it conveys a distinct role in downstream responses. Surprisingly, in *Lotus japonicus* we found that CS mutants showed reduced rhizobial infection and nodule number, which can be explained by disturbed long-distance N-signalling attributed to dysfunctional barrier formation. Moreover, we found that these mutants have disturbed CS formation in the nodule vascular endodermis, which represents a new model to elucidate the role of apoplastic barriers in nodule functioning.

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ORAL PRESENTATION

O 59

NITROGENASE IS THE MAIN TARGET OF A *RHIZOBIUM LEGUMINOSARUM* SMALL HEAT SHOCK PROTEIN

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Rhizobium leguminosarum bv. viciae (*Rlv*) is able to fix N_2 in symbiosis with some of the legumes of the Inverted Repeat-Lacking Clade group. These plants produce antimicrobial peptides (NCR) that induce the differentiation of vegetative cells into bacteroides. In addition to the NCRs, the bacteroids must be able to handle other stresses within the nodule (microaerobiosis, oxidative burst, etc.). Comparative proteomic studies of bacteroids induced by *Rlv* UPM791 strain in pea (*Pisum sativum*) and lentil (*Lens culinaris*) plants identified about 100 proteins with host-dependent expression[1]. Among them, we identified small Heat shock proteins (sHsp), which act as chaperones stabilising other partially denatured proteins in response to different types of stress. The aim of this work is to study the functional role of the sHsp_252 protein, a stress response protein overexpressed in pea bacteroids, in the *Rhizobium*-legume symbiosis.

The results obtained indicate that sHsp_252 is required to reach maximum levels of shoot dry weight and fixed nitrogen in pea plants. The promoter region of the *shsp_252* gene contains two anaerobic boxes, and regulation analysis by transcriptional fusions to *gusA* gene has shown that *shsp_252* is expressed under microaerobic conditions in a transcription factor FnrN-dependent manner. In addition, controlled induction experiments indicate that sHsp_252 offers cell protection against oxidative stress. Copurification analysis of sHsp_252 from pea bacteroids has identified NifD, NifK and NifH as the main targets for sHsp_252 together with another sHsp with which it could form heterooligomers. These results, along with the fact that rhizobia are atypical bacteria for harboring a large number of *shsp* genes in their genome[2], indicate that these proteins might play an important role in the adaptation of the bacteria for optimal nitrogen-fixing symbiosis inside the host.

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ORAL PRESENTATION

O 60

THE RESOLUTION OF CONDITIONAL SANCTIONING IN THE PEA *RHIZOBIUM LEGUMINOSARUM* SYMBIOSIS

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Westhoek et al., (2021)[1] demonstrated that the pea *Rhizobium leguminosarum* symbiosis is regulated by conditional sanctioning of inefficiently fixing strains. In this way nodules containing intermediately N_2 fixing rhizobia (50% of the wildtype rate) are either sanctioned or not depending on its co-inoculant. i.e. they will be sanctioned when competing nodules contain a wild-type strain but won't be sanctioned when competing nodules contain a strain incapable of fixation.

When inoculated at high concentrations some nodules are formed containing two strains of varying fixation ability. Whether the plant can sanction the strains within a mixed nodule separately is a matter of debate. By studying mixed nodules, we have demonstrated that within a nodule, plants are unable to sanction cells containing inefficient strains. We have demonstrated this in two ways, using flow cytometry we have shown that there is no significant difference in the proportion of efficiently and inefficiently fixing bacteria within a mixed nodule as we would expect to see in cellular sanctioning. We have also used confocal microscopy to investigate the cells within a mixed nodule and have found no difference in the health or morphology between cells infected with either strain up to forty-two days post infection. We have observed, however, that independent of the strain plant cells begin to undergo apoptosis after thirty-five days. This is indicative of a nodule wide sanctioning.

We have then gone on to show that sanctioning is a systemic process. This was achieved through the development of a split roots protocol. Roots are placed into separate pots and inoculated with different strains of varying fixing ability. In this split root system, we still see the same nodular sanctioning phenotype observed previously despite this separation. We conclude that the sanctioning of inefficiently fixing strains occurs at the nodular level, that this sanctioning is conditional on the efficiency of the co-inoculated strains. Furthermore, this sanctioning is controlled by a global and systemic regulatory system.

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ORAL PRESENTATION

O 61

NEGATIVE REGULATION OF SYMBIOTIC NITROGEN FIXATION BY A DEFENCE-RELATED MOLECULAR MECHANISM

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Legumes accommodate symbiotic rhizobacteria within plant cells of special organs, the root nodules, where rhizobia bind elementary nitrogen from the air. As a result of this symbiosis, cultivated legumes are able to provide themselves and subsequent rotation crops with nitrogen, reducing requirements for environmentally and economically costly mineral nitrogen fertilization. The past two decades of research on *Rhizobium*-legume interactions have provided unique insights into genetics and molecular biology of nodulation[1]. However, relatively little is known about the genetic mechanisms controlling or limiting the actual nitrogen fixation and symbiotic efficiency. Plants have evolved diverse strategies to limit microbes through transcriptional activation of antimicrobial molecular mechanisms. It is conceivable that genes supporting such microbial control have been co-opted to control bacterial symbiosis[2]. We hypothesized that negative regulators of symbiosis may be derived from ancestral defence genes through gene duplication and subsequent neo-functionalisation. Screening transcript levels of *Medicago truncatula* defence associated genes during the symbiosis with *Sinorhizobium meliloti* we indentified the symbiosis-specific upregulation of the β-*Glucan-Binding Protein 1* (*GBP1*) gene. *GBP* genes encode dual domain proteins with glucan-binding and hydrolytic activities towards microbial β-1,3/1,6-glucans[3, 4]. The Medicago genome encodes twelve members of the GBP family. Most of them are upregulated upon challenge with the pathogenic fungi and oomycete or by treatment with pathogen-associated molecular patterns such as flagelin or laminarin (a branched glucan, structurally similar to glucans from cell walls of filamentous pathogens). More detailed expression analysis shows that symbiotic specific induction of the *GBP1* gene is dependent on Nod factor perception and NIN-mediated transcription regulation. This suggests that activation of *GBP1* during interactions with rhizobia is a part of the host symbiotic program. Genetic deregulation of *GBP1* expression showed that GBP1 negatively regulates nitrogen fixation depending on the microsymbiont efficiency. More over, inactivation of GBP1 increases nitrogen fixation without affecting nodule numbers providing inroads for engineering legumes with increased productivity for sustainable nitrogen provision.

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ORAL PRESENTATION

O 62

INOSITOL PYROPHOSPHATES – POTENTIAL REGULATORS OF PLANT ROOT ENDOSYMBIOSES

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Tight regulation of nutrient homeostasis is vital for every cell. Plants have evolved elaborate systems to sense and signal extracellular and intracellular e.g. phosphate levels and to regulate cellular nutrient concentrations. Most land plants establish Arbuscular Mycorrhiza (AM) with phosphate-acquiring fungi, and selected members of the Fabales, Fagales, Cucurbitales and Rosales engage in root nodule symbiosis with diazotrophic bacteria[1,2]. While many genes involved in symbiont perception and subsequent genetic reprogramming have been well characterized, the signalling events that take place between the plasma membrane and the nucleus and the signalling hubs connecting symbiosis with nutrient homeostasis remain largely obscure. Plant phosphate homeostasis is regulated by inositol pyrophosphates (PP-InsPs). PP-InsPs bind to SPX domains - selective high-affinity PP-InsP receptors – and mediate the interaction of SPX and PHR-like transcription factors, thereby regulating the expression of phosphate starvation-induced genes[3]. There is accumulating evidence that phosphate homeostasis and AM signalling are interconnected *via* SPX and PHR[4-6]. Moreover, there is a direct link between phosphate and nitrogen homeostasis employing SPX. When SPX is degraded, PHR and NIN-like proteins migrate to the nucleus, where they activate phosphate starvation- and nitrate-induced genes, respectively, and coordinate in concert phosphate and nitrate utilisation[7]. It is our goal to scrutinize the role of PP-InsP ligands and putative precursors during symbioses and nutrient homeostasis in *Lotus japonicus* and thus to illuminate the interplay of these different plant strategies to overcome nutrient limitations.

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ORAL PRESENTATION

O 63

WHERE ARE NOD FACTORS COMING FROM? INVESTIGATING THE DISTRIBUTION AND FUNCTION OF LIPO-CHITOOLIGOSACCHARIDES IN BACTERIA AND FUNGI

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Nod factors produced by rhizobia were identified as lipo-chitooligosaccharides (LCOs) more than 30 years ago. These LCOs are required for intracellular infection and nodule organogenesis in most rhizobia-legume associations. The nature of substitutions on the LCO backbone is a significant determinant of host specificity in this symbiosis. About ten years ago, LCOs were also identified as produced by arbuscular mycorrhizal fungi. LCOs across rhizobia and fungi partly explain why several plant genes required for LCO signal transduction and involved in the common symbiotic pathway in legumes affect both root nodulation and mycorrhizal associations.

More recently, we demonstrated that LCOs are produced not only by arbuscular mycorrhizal fungi but by many fungi across the fungal kingdom (zygomycetes, ascomycetes, and basidiomycetes). These fungi exhibit various lifestyles as symbionts, pathogens, and saprotrophs; most are not associated with plants. We also demonstrated that many fungi respond to these LCOs in various dose-dependent ways, suggesting that LCOs could be fungal quorum-sensing-like molecules. We will briefly report progress on the dissection of LCO perception and signaling in *Aspergillus fumigatus* (ascomycete) and *Laccaria bicolor* (basidiomycete).

The presence of genes potentially allowing the production of LCOs has been reported in Frankia Cluster II bacteria. We identified such genes and demonstrated LCO production in several other Gram-positive Actinomycetota and Bacillota bacteria that do not fix nitrogen or seem to associate with plants. We will present our current model for the origin and role of LCO production in fungi and bacteria and its implications on the origin of mycorrhizal and root nodule symbioses.

ORAL PRESENTATION

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ROLE OF PHOSPHORUS IN THE LEGUME RHIZOSPHERE INTERFACE AND CONSEQUENCES ON SYMBIOTIC PERFORMANCE AND YIELD

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Phosphorus (P) is an important nutrient which is highly needed for a better crop growth and yield production. However, the availability of P is often low in most soils and that cropping systems, particularly legumes, in low-P agro-systems require, among others, supplemental P to maximize their yield. In addition, biological strategies based on plant- and microbe-potentialities have been increasingly adopted as a promising way to improve P use efficiency (PUE) in N_2-fixing legumes. It is now believed that a rational use of mineral P fertilizers alongside agriculturally profitable biosystems (e.g., cropping systems, beneficial microbes, etc.) may contribute to a productive and sustainable agriculture.

In this context, greenhouse- and field-based research investigations have mainly been focused on studying PUE in several grain legumes (e.g., common bean, faba bean, soybean sole crop or inter-crops). Overall, findings showed that rhizosphere modifications including variations in root architectural traits, nodulation, root and nodule stratification across soil layers, rhizosphere acidification, induction of specific genes (e.g., P-hydrolyzing phosphatases, etc.) are all involved in PUE. Research on N_2-fixing legumes has reported various rhizosphere biochemical and architectural modifications associated to a higher PUE. Stimulation of rooting system, nodulation, nodule respiration, as well as enzyme activities and gene expression of several phosphatases were found to be involved in PUE and plant growth. Belowground interactions in legume-based cropping systems such as the grain legume-cereal intercrop were also reported to improve plant P acquisition. Increased biomass, length, and surface area of roots, efficient rhizobial symbiosis, increased P hydrolyzing acid phosphatases, higher root microbial diversity and grain yield were found to be associated with higher PUE in different cereal-legume intercrops. In addition, importance of P-solubilizing and N_2-fixing bacteria was highlighted through a series of inoculation experiments under different P regimes (soluble vs insoluble).

As per current knowledge, it is highly important to continue understanding how legume crops and associated microbes may achieve an efficient use of P under BNF conditions. Such a biological approach needs to be further optimized for a better use of mineral resources such as P, crop productivity and stress tolerance.

ORAL PRESENTATION

O 65

ESTABLISHING EFFICIENT BIOLOGICAL NITROGEN FIXATION AS A BREEDING TARGET IN VICIA FABA

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Faba bean (*Vicia faba*) is a high-protein, high-yield grain legume crop, which makes it a promising candidate for replacing imported plant protein in the EU. It is predominantly cultivated as an organic crop in Europe and can benefit from the use of rhizobial inoculants to enhance economic yields. However, to achieve maximum benefits, the rhizobial inoculants must be effective in providing the plant with fixed nitrogen and competitively colonize the nodules in the presence of native rhizobia. With the aim of finding faba bean varieties that can choose effective N_2-fixing rhizobia, we created a Faba bean database by collecting genotypic and phenotypic information from ~255 lines[1] and a Soil database using data from 12 soils across five European countries, which includes physical and chemical information, allowing us to create unique soil profiles for each of the 12 soils. We trapped and isolated ~1200 novel rhizobial strains using faba bean plants[2], which resulted in the creation of our Rhizobial database. Using the rhizobial identification "Plasmid-ID" method[3], we simultaneously monitored the interaction of 452 rhizobial strains in 213 faba bean lines in greenhouse assays. Plant growth was evaluated under three conditions: i) rhizobial inoculation (+R), ii) full chemical fertilisation, including NH_4NO_3 (+N), and iii) nitrogen starvation (-N). Approximately 2000 plants were harvested, and Plasmid-ID sequencing was performed to identify the rhizobial strains present in each inoculated plant.

We investigated the relationship between biomass production and rhizobia relative abundance data using linear mixed-effect models. To analyse the inter-strain interactions, we conducted network analysis. Using regression-based methods, we calculated the interactions for each isolate that played a significant role in colonising the community.

Here, an integrated approach is presented by studying genotype/phenotype associations in diverse faba bean cultivars and identifying their best rhizobia match, with the aim of establishing efficient biological nitrogen fixation as a breeding target.

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ORAL PRESENTATION

O 66

GENOME-WIDE IDENTIFICATION OF COLONISATION DETERMINANTS IN *RHIZOBIUM LEGUMINOSARUM* USING RB-TNSEQ

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Plant growth promoting bacteria must persist in the rhizosphere and colonise the roots of their host plant in order to exert their beneficial effects. *Rhizobium*-legume symbioses are one of the best characterised beneficial interactions due to their potential to alleviate our reliance on nitrogen fertilisers. Despite this, most studies on *Rhizobium* spp. have focused on its symbiotic lifestyle as an endosymbiont in root nodules and therefore the initial stages of rhizosphere growth and root colonisation remain relatively under characterised[1]. The multifactorial nature of bacterial growth in the rhizosphere and root colonisation means that transposon insertion sequencing techniques such as random-barcode transposon-site sequencing (RB-TnSeq) provide a unique opportunity to study gene function at the whole genome level[2]. Through incorporation of a random DNA barcode into transposons RB-TnSeq can be used to assay mutant fitness in a high throughput manner[3].

In this work RB-TnSeq was used in the model rhizobial strain, *Rhizobium leguminosarum* bv. *viciae* 3841, to characterise how plant species and bacterial competition affect growth in the rhizosphere and colonisation of host legumes, a non-host legume and a non-legume. A core set of 97 genes were required for growth in all plant rhizospheres and a further 69 genes commonly contributed to root colonisation. Analysis of these genes revealed that rhizosphere growth and root colonisation required the synthesis of compounds (amino acids, ribonucleotides and cofactors), alteration of metabolic function, adaptation to various stresses (such as changes in osmolarity) and sensing of external stimuli coupled with modification of gene expression. Additionally, chemotaxis and flagella-mediated motility were common prerequisites for root colonisation. Notably, many genes showed plant-specific dependencies highlighting the significant adaption required to different plant rhizospheres. This work provides a greater understanding of factors promoting rhizosphere fitness and root colonisation in plant-beneficial bacteria.

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ORAL PRESENTATION

O 67

IS ETHYLENE SIGNALING REQUIRED FOR DROUGHT STRESS RESPONSES IN *MEDICAGO TRUNCATULA*?

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Ethylene is one of the plant hormones generally associated with stress responses such as pathogen attack and wounding. However, the role of ethylene in drought stress remains unclear. Previous work often subjected the plants to extremely drying conditions (i.e., using excised leaves or plants exposed to the air). Thus, the responses observed may not be related to what occurs in the field, where water deficit occurs in a more progressive manner.

Here we took advantage of the *Medicago truncatula* ethylene-insensitive mutant *sickle* (*skl*)[1], which expresses a truncated version of the MtEIN2 protein, to test whether altered ethylene signaling has an impact on plant growth and response to progressive drought stress. Both *skl* and the wild-type genotype, A17, were able to grow fully based on symbiotic nitrogen fixation, presenting similar plant biomass, N content, and leaf chlorophyll levels. Hormonal profiling showed increased levels of 1-aminocyclopropane 1-carboxylate (ACC), the precursor of ethylene, under severe drought conditions, with *skl* showing the highest values. The levels of several cytokinins and other stress response hormones such as abscisic also showed a drought-induced accumulation, but this increase did not require an active ethylene signaling pathway. Our results show a complex regulation of drought responses with the crosstalk of multiple hormone pathways.

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ORAL PRESENTATION

O 68

ROLE OF FERRITIN(S) IN NODULE FORMATION AND THE SYMBIONT INDUCED STAY-GREEN EFFECT UPON DROUGHT

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The symbiotic relationship between nitrogen-fixing rhizobacteria and legumes play a crucial role in enhancing plant tolerance to drought stress[1]. Previous research has proven that this symbiosis triggers a phenotype known as symbiont induced stay-green (SISG), which delays leaf senescence in response to drought and facilitates quicker recovery from desiccation[2]. Ferritin, an Fe-storage and distribution protein, known to be involved in drought stress[3] and leaf senescence[4], seemed to play a major role in this stay-green phenotype. Their levels were enhanced during symbiosis compared to non-symbiotic *Medicago truncatula* (Jemalong) plants[2,5].

Here, we show that Ferritins are additionally involved in nodule formation. Knockdown mutants exhibited reduced nodulation while function was retained. Hence, ferritin supports the formation of the symbiotic relationship and symbiosis, vice versa, induces the levels of Ferritin. As a consequence, ferritins are not only regulated by rhizobia but are also regulating symbiosis. In order to decipher the dynamics of the different isoforms of Ferritin in this relation(s), we further analysed the absolute amount of ferritin isoforms in leaves of *M. truncatula* wildtype R108. For this, we used the Mass Western approach and compared protein levels upon symbiosis and drought. The results will be discussed in this poster.

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ORAL PRESENTATION

O 69

ROLE OF INTER-ORGAN SYSTEMIC SIGNALING IN THE CONTROL OF THE RHIZOBIUM–LEGUME SYMBIOSIS BY THE WHOLE PLANT NITROGEN DEMAND

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Symbiotic nodules formed on legume roots with rhizobia fix atmospheric N_2. Bacteria reduce N_2 to NH_4^+ that is assimilated into amino acids by the plant. In return, the plant provides photosynthates to fuel the symbiotic nitrogen fixation. Symbiosis is tightly adjusted to the whole plant nutritional demand and to the plant photosynthetic capacities, but regulatory circuits behind this control remain poorly understood. The use of split-root systems combined with biochemical, physiological, metabolomic, transcriptomic, and genetic approaches revealed that multiple pathways are acting in parallel [1–6]. Systemic signaling mechanisms of the plant N demand are required for the control of nodule organogenesis, mature nodule functioning, and nodule senescence. N-satiety/N-deficit systemic signaling correlates with rapid variations of the nodules sugar levels, tuning symbiosis by C resources allocation[4]. These mechanisms are responsible for the adjustment of plant symbiotic capacities to the mineral N resources. On the one hand, if mineral N can satisfy the plant N demand, nodule formation is inhibited, and nodule senescence is activated[4,5]. On the other hand, local conditions (abiotic stresses) may impair symbiotic activity resulting in plant N limitation. In these conditions, systemic signaling may compensate the N deficit by stimulating symbiotic root N foraging [2–4]. In the past decade, several molecular components of the systemic signaling pathways controlling nodule formation have been identified, but a major challenge remains, that is, to understand how they contribute to the whole plant phenotypes [6]. Less is known about the control of mature nodule development and functioning by N and C nutritional status of the plant, but a hypothetical model involving the sucrose allocation to the nodule as a systemic signaling process is proposed [4,6]. Altogether these data highlight the importance of organism integration in the biology of rhizobium-legume symbiosis.

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POSTER
Symbiotic Signaling

P 1

REDOX-SENSITIVE FLUORESCENT BIOSENSORS DETECT INTRACELLULAR REDOX VARIATIONS IN *SINORHIZOBIUM MELILOTI* UNDER FREE-LIVING AND SYMBIOTIC LIFESTYLES

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Reactive Oxygen Species (ROS) are key signaling molecules that control setup and functioning of the nitrogen-fixing symbiosis between *Sinorhizobium meliloti* and *Medicago truncatula*. Changes in ROS concentration have been detected from the earliest stage of interaction up to mature nodules, and the bacterial antioxidant defense has been shown to be critical for an efficient symbiosis[1]. The main objective of this study was to analyze variations in the cytoplasmic redox state of the bacterial partner during symbiosis. We took advantage of two redox biosensors, roGFP2-Orp1 and Grx1-roGFP2, consisting of a redox-sensitive green fluorescent protein (roGFP2) fused to the thiol peroxidase Orp1 (specific to hydrogen peroxide, H_2O_2) and the glutaredoxin Grx1 (related to glutathione redox buffer), respectively[2]. We measured dynamic changes of intracellular H_2O_2 and glutathione redox potential in *S. meliloti* expressing roGFP2-Orp1 and Grx1-roGFP2. Under free-living conditions, spectrofluorimetric measurements showed that the biosensors are suitable to monitor the bacterial redox state in real-time, after H_2O_2 challenge and in different genetic backgrounds. *In planta*, flow cytometry and confocal imaging experiments allowed the determination of sensor oxidation state in bacteria within nodules. These cellular studies establish the existence of an oxidative shift in the redox status of *S. meliloti* when bacteria differentiate into nitrogen-fixing bacteroids. Our findings open up new possibilities for *in vivo* studies of redox dynamics during N_2-fixing symbiosis[3].

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P 2

SYSTEMIC SIGNALLING IN LEGUME-RHIZOBIA SYMBIOSIS

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The partnership between legumes and N-fixing rhizobia requires a complex exchange of systemic signals that function to regulate nodulation. Some of the components of this signaling network are still unidentified or poorly studied in legumes. In this work we have used specific mutants from the model legume *Lotus japonicus* to study three processes related to nodulation and the N status of the plant: 1) Asparagine biosynthesis. Mutants in a gene encoding for a specific isoform of asparagine synthetase indicates a different role of this enzyme under symbiotic or non-symbiotic conditions. Differential growth ratios of the mutant compared to the WT are paralleled by distinctive metabolomic profiles. 2) Flavonoid and isoflavonoid biosynthesis. Analysis of *L. japonicus* gene co-expression networks allowed the identification of genes encoding for MYB transcription factors that may regulate the biosynthesis of isoflavonoids. A combined transcriptomic and metabolomic approach suggests a role for specific MYB factors in the regulation of the biosynthesis of these compounds. 3) Glutaredoxins (GRX). Three genes encoding for different GRXs were shown to be more highly expressed in shoots and were downregulated in nodulated plants compared to non-nodulated plants grown on ammonium nitrate[1]. Specific mutants in one of these GRX show enhanced nodulation even in the presence of external nitrogen, thus suggesting a role for this protein in the systemic regulation of nodulation.

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P 3

HOSPHORYLATION-BASED FUNCTIONAL SWITCH OF AN E3 LIGASE BETWEEN K48- AND K63-LINKED UBIQUITINATION REFINES RECEPTOR LEVELS IN LEGUME NODULATION

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A precise control of receptor levels is essential for activating cellular signaling transduction in response to specific ligand. However, such mechanism regarding plant's nodulation factor (NF) receptors levels in response to rhizobial NFs remains to be elucidated. Here, we identify that NFR-Interacting RING-type E3 ligase1 (NIRE1) plays a dual role in regulating NFR1/NFR5 homeostasis to optimize rhizobial infection and nodule development in *Lotus japonicus*. NIRE1 associates with both NFR1 and NFR5. NIRE1 promotes the degradation of NFR1/NFR5 through K48-linked polyubiquitination before rhizobia inoculation. While, upon rhizobia inoculation, NIRE1 is phosphorylated by NFR1 at a conserved residue Y109 and then switches its function to mediate K63-linked polyubiquitination to stabilize NFR1/NFR5. Overall, the data reveal a novel mechanism in which NFR1/NFR5 protein levels are dynamically regulated by a single E3 ligase via phosphorylation-dependent functional switch to mediate differential linkages of ubiquitin in the nodule symbiosis.

P 4

DUAL-LOCK REGULATION OF SYMBIOSIS RECEPTOR KINASE (SYMRK) DIRECTS RHIZOBIAL INVASION AND COLONIZATION DURING ROOT NODULE SYMBIOSIS

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Symbiosis Receptor Kinase (SYMRK) is indispensable for root nodule symbiosis and is functionally conserved within the nitrogen-fixing clade. It is a member of LRR-RLK-I subfamily where the ectodomain (ectoD) has a malectin-like domain (MLD) connected to leucine-rich repeats (LRRs) through a hinge region containing a conserved GDPC motif. In SYMRK this motif is required for MLD shedding in ectoD. Within the hinge region of the cytoplasmic kinase domain (KD), all RLKs have a conserved Tyr in the gatekeeper position. SYMRKs are distinguished by having a conserved Pro in the gatekeeper+1 position whereas all other MLD or MLD-LRR RLKs have Glu/Asp. KD-hinge substitutions in gatekeeper (**Y670F**) or gatekeeper+1 (**P671E**) positions restricted SYMRK in a basal auto-activation state [1] and also inhibited MLD shedding. Additionally, the hinge substitution mutants of both KD (**Y670F, P671E**) and ectoD (**P388L**) were phenotypically similar, where rhizobial progression was hindered at the epidermal-cortical barrier [2]. Intriguingly, ectopic expression of MLD allowed complete crossover of rhizobial infection beyond the epidermal cortical barrier in both ectoD and KD-hinge SYMRK mutants. These observations indicated an inside-out signal derived from optimally phosphorylated SYMRK-KD to be required for activating the cleavage of MLD in SYMRK ectoD. We reveal another layer of regulation of SYMRK by demonstrating kinase activation and ectodomain shedding to develop a 'dual-lock' for allowing rhizobial invasion through the epidermal cortical barrier.

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P 5

THE B-TYPE RESPONSE REGULATOR GMRR11D MEDIATES SYSTEMIC INHIBITION OF SYMBIOTIC NODULATION

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Symbiotic nodulation is imperative for the survival and productivity of soybean. Under low nitrogen conditions, soybean plants perceive lipochitooligosaccharide signals (nodulation factors [NFs]) released from rhizobia through NF receptors to initiate a molecular cascade to trigger nodulation[1, 2]. Symbiosis is energy consuming; therefore, soybean plants develop an autoregulation of nodulation (AON) to control the homeostasis of nodulation. In AON, nodulated roots produce peptides GmRIC1 (rhizobia induced CLAVATA3/ESR [CLE] peptide 1) and GmRIC2 that are transported from roots to shoots to activate their leucine-rich-repeat receptor kinases GmNARK (soybean nodulation autoregulation receptor kinase), and the GmNARK then inhibits further nodulation[3, 4, 5, 6, 7]. However, the mechanism by which GmNARK suppresses nodulation remains largely unclear. It has long been proposed that shoot-controlled nodulation acts downstream of cytokinin (CK) signaling-mediated activation of nodule initiation, but the mechanisms are not fully understood[8, 9]. In this study, we conducted a systemic study using a combinatorial approach to answer the above scientific questions. We show that a B-type response regulator of CK, GmRR11d, mediates systemic inhibition of nodulation by AON and GmNARK in roots. *GmRR11d* is induced by rhizobia and low level cytokinin. Genetic evidence show that *GmRR11d* negatively regulates soybean nodulation. In addition, we found that GmRR11d can directly bind to *GmNIN1a* promoter to repress its expression, and it can also suppress the transcriptional activity of GmNSP1 on *GmNIN1a* to inhibit NF signaling that reduces soybean nodulation. Furthermore, GmRR11d positively regulates cytokinin response and its binding on the *GmNIN1a* promoter is enhanced by cytokinin. Intriguingly, rhizobial induction of *GmRR11d* and its function are dependent upon GmNARK. Thus, GmRR11d governs a transcriptional program associated with nodulation attenuation and cytokinin response activation essential for systemic regulation of nodulation. Together, our findings elucidate the molecular mechanism by which GmNARK suppresses further nodulation by simultaneously repressing NF signaling and increasing CK sensitivity in soybean.

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P 6

PRELIMINARY CHARACTERIZATION OF THE *L. JAPONICUS* TETRASPANIN FAMILY

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Plant tetraspanins (TPSs) are scaffolding proteins associated with root radial patterning, biotic and abiotic stress responses, cell fate determination, lateral root meristem formation and hormonal regulation. They have been proposed to be involved in the mechanisms generating reactive oxygen species responses (ROS) and in vesicular trafficking and polarized growth. TPSs are also considered as specific markers of exosomes, vesicles derived from the exocytic multivesicular bodies (MVB) that can load important molecules such as lipids, proteins, messenger RNA, and microRNAs and play important roles in cell-to-cell communication in animal and plant cells. These vesicles represent crucial tools to overcame organism boundaries as these have been reported to mediate plant pathogen interactions [1]. Furthermore, the formation of extracellular vesicles has been recently reported during the plant-mycorryza symbiotic interaction at the interface between arbuscule mycorryza and root invaded cells throughout the arbuscule lifespan [2].
The phenotypes described above are consistent with a potential involvement of TPSs during different steps of the symbiotic interaction between legume plants and rhizobia.
In a preliminary study, using conserved sequences of *A. thaliana* and *Oryza sativa* as queries, we have predicted members of the tetraspanin (*TSP*) gene family in *L. japonicus*. These sequences were further screened for several conserved residues and domains leading to the identification of 12 putative TSP sequences in *L. japonicus* sharing from 31% to 40% identity with the query sequences. The temporal and spatial expression profiles during the N_2-fixing symbiotic interaction will be presented. This preliminary characterization of the *L. japonicus TSP* family allowed to identify potential candidates playing roles during the early steps of symbiotic interaction and a reverse genetic approach through the isolation of knock out LORE1 insertion mutants is in progress.

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THE EFFECTORS OF THE T6SS IN *RHIZOBIUM ETLI* MIM1 BENEFIT BACTERIAL COMPETITION

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The type VI secretion system (T6SS) is a nanosyringe of some Gram-negative bacteria that injects proteins called effectors into other bacteria or eukaryotic cells. Most effectors have antibacterial activity. T6SS structural genes are present in many sequenced *Rhizobium* spp.[1], but their relevance is not well understood. Previously we found a positive effect of *Rhizobium etli* Mim1 (ReMim1) T6SS on bean symbiosis[2]. However in this abstract we show that the potential effectors in the T6SS cluster are not required for effective nodulation with beans. ReMim1 T6SS genes corresponding to potential effectors were divided in three gene modules, and their expression was studied by RT-qPCR. In these experiments, low levels of expression of genes from the three modules were observed under both free-living and symbiotic conditions. We analyzed T6SS expression, by fusing a putative promoter region between the *tssA* and *tssH* genes in both orientations to a reporter gene. Both fusions were active in free-living cells and in symbiosis. Two proteins from the first module (Re78 and Re79) were expressed in *E. coli* and found to behave as a toxic effector/immunity pair (E/I) respectively. The harmful action of Re78 takes place in the periplasmic space of the target cell. Deletion of this E/I pair resulted in reduced competitiveness for bean nodule occupancy and lower survival in the presence of the wild-type strain. Therefore, a better understanding of the role of this secretion system can be very useful to select competitive rhizobia for inoculants and biocontrol agents.

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UNDERSTANDING THE MOLECULAR BASIS OF *SINORHIZOBIUM FREDII* HH103-SOYBEAN COMPATIBILITY CONFERRED BY BACTERIAL SECRETED PROTEINS

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To establish N-fixing symbiosis, rhizobial bacteria must bypass the plant immune system[1]. Similar to plant pathogenic bacteria, many rhizobial strains suppress plant defence delivering effector proteins into the host cell via Type III Secretion System (T3SS)[2]. However, protein effectors cannot always suppress plant immunity. Plants have evolved the presence of host resistance (*R*) genes able to recognize directly/indirectly the T3SS effectors, blocking the bacterial infection through the activation of the effector-triggered immunity (ETI) response[3]. The molecular mechanisms involved in recognising rhizobial effectors are poorly understood.

Sinorhizobium fredii HH103 is known as a 'promiscuous strain' and it is symbiotically compatible with the agronomically-important soybean William 82 cultivar. Our preliminary results showed that the HH103 strain cannot establish symbiosis with all soybean cultivars. For example, the wild-soybean CH2 cultivar (PI407288) inoculated with HH103 did not form any nodules. We hypothesized that HH103 gain and/or loss of nodulation phenotypes might be driven by evolution processes through the recognition of T3SS secreted effectors. We constructed loss-of-function mutants in every T3SS effector gene of HH103. Nodulation assays showed both effector-dependent gain or loss of nodulation phenotypes in both William 82 and CH2 cultivars, suggesting that T3SS effectors are involved in rhizobia-soybean symbiotic compatibility. We will perform Genome Wide Association Studies, Immunoprecipitation coupled with Mass-spectrometry and Structural Biology approaches to study the role of HH103 T3SS effectors in the plant host cell.

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P 9

MECHANISMS OF BACTERIAL PRIMARY ATTACHMENT TO PLANT ROOTS UNDER DIFFERING PH CONDITIONS

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The initial stages of primary symbiotic bacterial attachment to plant roots are poorly characterised compared to later stages of symbiosis, such as root nodule formation or the functioning of the nitrogenase complex [1]. This study aims to characterise key mechanisms of *Rhizobium leguminosarum* bv. *viciae* attachment to *Pisum sativum* roots under different pH conditions, focusing on the regulation exerted by the attachment repressor *praR* [2]. Attachment at acidic, basic, and neutral pH was investigated by the stepwise interrogation of the repressor *praR* regulatory system using RNA sequencing, promoter fusions to study gene expression, and *lux*-based bioreporter attachment assays of mutants. This experimental set up included those genes essential for primary attachment and those differently regulated according to pH and bacterial population density [2, 3, 4]. Our results show that the mutation of *praR* at neutral pH causes an 161% increase in primary root attachment and an 163% increase in root colonisation 7 days post inoculation. Of particular interest is the gene RL0149 which is repressed by *praR* and shares 55% protein sequence similarity and 38% identity with *praR*. These two are XRE family transcriptional regulators and may cross-regulate mechanisms of primary attachment depending on pH. Genes regulated in a pH-dependent manner in this system include the autoaggregation proteins *rapA2, rapB,* and *rapC,* and the cadherin domain-containing calcium-binding glycoproteins *cadA* and *cadB*. It had been proposed that an extracellular calcium-binding protein termed rhicadhesin is a dominant mediator of rhizobial attachment via an unknown plant receptor at pH 7.5 [5]. However, neither the protein nor its gene have been isolated or identified to date. We hypothesize that the effect assigned to this hypothetical rhicadhesin protein may be indeed the combined effect of the adhesins that are cross-regulated by the *praR*-RL0149 system.

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FROM RHIZOSPHERE TO ROOT NODULE: UNRAVELLING THE GENETIC PATHWAYS THAT DIFFERENTIATE INTER- AND INTRACELLULAR INFECTION OF LEGUMES BY RHIZOBIA

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The symbiotic partnership between rhizobia and legumes culminates in the formation of root structures, termed nodules, where the rhizobia reside and provide fixed nitrogen for the host in exchange for energy. Nodulation requires a signalling dialogue between the symbiont and host, following which the bacteria must pass the epidermal barrier of the plant to reach the root cortex and infect the nodule primordia. There are two primary routes of infection: intracellular infection where the bacteria traverse through root hair infection threads (ITs), or intercellular infection where bacteria enter between epidermal cells, or through fissures (crack entry). Approximately 75% of legume species are infected via the intracellular mechanism, a sophisticated and genetically complex pathway subjected to intensive research efforts. Intercellular infection, however, is considered an ancient and primitive pathway and remains poorly understood comparatively. Some legumes are capable of both IT-based and intercellular infection, but usually requires a conditional change to switch mechanism. The bacterium *Rhizobium* sp. IRBG74 was isolated from the legume *Sesbania cannibina,* which it can infect by both pathways, depending on the flooding conditions of the roots. IRBG74 was recently shown to form nodules on the model legume *Lotus japonicus* exclusively through the intercellular mechanism. It formed effective nodules on *Lotus*, however, the nodulation kinetics showed a substantial delay in onset (approx. 14 dpi) relative to the *L. japonicus* symbiont *Mesorhizobium japonicum* R7A (7 dpi), and no ITs were observed. Recently we isolated an IRBG74::Tn*5* transposon mutant from *L. japonicus* that displayed not only similar nodulation kinetics to R7A (approx. 8 dpi), but also induced full length ITs. Aside from the single Tn*5* insertion, the mutant and wild-type IRBG74 strains share an otherwise isogenic background. Therefore, we can now compare the distinct pathways of the intra- and intercellular symbiotic infection mechanisms directly in the model legume, *L. japonicus.* Comparative analyses are underway that will identify both the plant and bacterial molecular elements that contribute to this switch in the infection strategy.

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GENETIC AND FUNCTIONAL DIVERSITY OF *LOTUS* MALECTIN-LIKE DOMAIN LEUCINE-RICH REPEAT RECEPTOR KINASES IN ROOT ENDOSYMBIOSIS

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Arbuscular Mycorrhiza (AM) and Root Nodule Symbiosis (RNS) both require cortical and epidermal signalling modules to establish intracellular accommodation of the microbial symbiont. Two receptor kinase families are required for epidermal infections, including the malectin-like domain leucine rich repeat receptor kinase Symbiosis Receptor Kinase (SymRK) [1, 2, 3, 4]. While SymRK is indispensable for epidermal infection in AM and RNS, we observed that tissue-specific expression of *SymRK* in the root epidermis was sufficient to restore AM and RNS in a *symrk* mutant. Based on this observation, we hypothesized that SymRK-like receptors could be involved in cortical signalling in root endosymbiosis. To explore this hypothesis, we identified a family of SymRK Homologous Receptor-Like Kinases (SHRKs) [5]. and investigated their roles in AM and RNS.

In a preliminary analysis of root endosymbiosis phenotypes of *shrk LORE1* insertion mutants, we found that their phenotypes differ from that of *symrk*. In this work, we aim to dissect the evolutionary, functional and molecular relationship between SymRK and SHRKs.

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P 12

MTANNEXIN1 IS REQUIRED FOR THE DEVELOPMENT OF FULLY FUNCTIONAL NODULES

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MtAnnexin1 (*MtAnn1*) encodes a *Medicago truncatula* calcium-and phospholipid-binding protein of the annexin family[1]. Its expression is induced in response to *Sinorhizobium meliloti* both at early stages in bacterial-inoculated roots and in developing nodules[2,3]. Notably, expression of *MtAnn1* transcription and translational protein fusions are strongly activated in outer cortical cells at early stages after root inoculation suggesting its involvement in the reprogramming of host cells for rhizobia infection[4]. MtAnn1 has possible roles in the regulation of ion conductance[5] and its contribution might thus be connected to symbiotic calcium signalling. With this in mind, we have investigated by *in vivo* microscopy the relationships between the spatio-temporal dynamics of an MtAnn1-GFP fusion and calcium spiking responses, imaged using a nuclear GECO calcium sensor[6] during early host cell reprogramming for rhizobial infection in WT and symbiotic mutants impaired for rhizobial infection. This study highlighted a close correlation between calcium spiking and MtAnn1-GFP dynamics in epidermal and outer cortex cells preparing for or undergoing infection, and revealed new genetic determinants of this process, notably Nodule Inception (NIN), that acts as a master regulator of *MtAnn1*. Strikingly, mutation in *MtAnn1* results in modification of Ca^{+2} oscillations in root hairs, reduced levels of rhizobia infection and impaired development of nodules.

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UNRAVELLING THE NON-CODING TRANSCRIPTOME OF *SINORHIZOBIUM FREDII* HH103

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Sinorhizobium fredii HH103 is a broad-host range rhizobium that can form nitrogen-fixing relationships with many legumes. The communication between the bacteria and the plant in this relationship is complex, involving several molecular signals from both parties. The bacterial signals include lipochitooligosaccharides (LCOs) or Nodulation Factors (NF), effector proteins secreted by the T3SS (Nops), and various surface polysaccharides (EPS, LPS, KPS, and GC).
Previous transcriptomic studies addressed the regulation of the production of these *S. fredii* HH103 symbiotic signals at the transcriptional level [1, 2, 3], but they did not consider the regulatory role of small RNAs (sRNAs). In this work, we present our first analysis of the non-coding transcriptome of *S. fredii* HH103. We have carried out an especial RNA-seq analysis (Cappable-seq) aimed to the determination of the transcriptional start sites (TSS) in this genome (13,072), that were assigned to four different types of transcripts: mTSS (mRNA), sTSS (sense sRNAs), asTSS (antisense sRNAs), tTSS (trans sRNAs).
One of the identified HH103 tTSS likely corresponds to a *trans*-sRNA (initially termed F6) encoded immediately downstream a *nod* box and its expression is strongly induced by the *nod*-gene inducing flavonoid genistein. Preliminary analyses revealed that F6 deletion resulted in a strong impairment of the interaction of HH103 with soybean, suggesting an important role for this trans sRNA for symbiosis with this legume.

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ARE GLUTAREDOXINS INVOLVED IN NODULATION?

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Glutaredoxins (GRXs) are small proteins of around one hundred amino acids that use glutathione as substrate. They constitute a broad group of proteins which are found in most organisms and, between other functions, they are involved in the maintenance of cellular redox homeostasis[1]. There are several GRXs that only appear in plants but little is known about them in relation to nodulation in legume plants[2]. Recent analysis in the model legume *Lotus japonicus* indicated that some of the genes encoding for GRXs were differentially expressed in plants growing under non-symbiotic or symbiotic conditions with *Mesorizhobium loti* [3], thus suggesting a possible role of them in nodulation. We will show the recent advances produced in our laboratory in the functional study of these GRXs through the characterization of *L. japonicus* LORE1 homozygous mutant plants.

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LEGUMES REGULATE SYMBIOSIS WITH RHIZOBIA VIA THEIR INNATE IMMUNE SYSTEM

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Nucleotide-binding leucine-rich repeat receptors (NLRs) are key components of the plant innate immune system, facilitating the detection and inhibition of invasive pathogenic organisms. Recent findings have broadened our understanding of their functionality, including novel roles in calcium signalling and NADase activity [1,2]. In the legume-rhizobia symbiosis, plant NLRs play key roles in partner compatibility by recognising proteins secreted by certain rhizobia and restricting symbiosis [3]. This has powerful implications for agriculture where rhizobia inoculum for nodulation is based on compatibility between the legume species and rhizobia strain. NLRs are therefore important targets for nodulation research; however, only a few have been functionally characterised and further work is needed to understand the scope of NLR activity in this symbiotic process. Drawing on recent findings, we outline currently known similarities between plant immunity and nodulation and discuss how legumes regulate symbiosis with rhizobia utilising the diverse NLR gene family.

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SINORHIZOBIUM MELILOTI NUTRITIONAL STATUS CHANGES DURING EARLY ROOT HAIR INFECTION

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In the model legume *Medicago truncatula,* as in most symbiotic Legumes[1], rhizobial infection occurs through the formation of tubular infection threads (ITs) in root hair cells. Polar tip growth of ITs is initiated and directed by the host plant[2], while rhizobia concomitantly progress and grow inside ITs[3]. Compared to a free-living state in the rhizosphere, enclosure of rhizobia in the IT apoplastic compartment probably triggers major changes in the bacterial lifestyle, notably regarding access to nutrients. To investigate if the nutritional status of rhizobia changes during IT growth, we developed advanced imaging tools in *Medicago*, enabling to live track the dynamics of nutrition-associated fluorescent protein fusions in individual *Sinorhizobium meliloti* cells within the IT. Phasins are proteins associated with poly-3-hydroxybutyrate (PHB) granules, which accumulate in *S. meliloti* cells under conditions of nutritional imbalance (excess carbon compared to other essential nutrients such as nitrogen and phosphorus)[4,5]. Live dynamics of fluorescent phasin protein fusions revealed the gradual accumulation of PHB granules along IT maturation, suggesting progressive bacterial nutrient limitation when immobilized in mature ITs. Co-imaging studies revealed a striking opposite labelling pattern for the YFP-ENOD11 plant apoplastic marker, preferentially labelling actively growing regions of ITs, and *S. meliloti* phasin fusions, rather labelling bacteria in mature parts of ITs. This suggests a possible association between rhizobia nutritional imbalance and progressive stiffening of the plant cell wall. Major questions remain about the source of this nutritional imbalance and how the plant affects it, which we are currently addressing using bacterial nutrient starvation (nitrogen or phosphorus) fluorescent reporters and plant mutants.

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A JUXTAMEMBRANE PROTEIN-PROTEIN INTERACTION MOTIF IN THE *LOTUS JAPONICUS* NFR5 INTRACELLULAR DOMAIN IS ESSENTIAL FOR ROOT NODULE SYMBIOSIS

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Legume-rhizobia root nodule symbiosis require the plant LysM receptor kinases Nod Factor Receptor 1 (NFR1) and NFR5 for perception of Nod factors and subsequent initiation of the symbiosis signaling pathway. Receptor signaling requires the catalytic activity of the NFR1 protein kinase, while the mechanistic role for the catalytic inactive NFR5 pseudokinase is less well understood. We have solved the crystal structure of the intracellular domain of the NFR5 receptor, including the juxtamembrane domain and the protein kinase domain. Structural analysis confirms NFR5 contains several diverged and truncated catalytic motifs, rendering the kinase catalytic inactive. The structure additionally revealed the juxtamembrane of NFR5 contains two alpha-helices, which form a solvent exposed hydrophobic motif. This hydrophobic motif resembles a known juxtamembrane dimerization motif in the well-studied Epidermal Growth Factor Receptor and minimal substitution mutations in the motif abolishes NFR5 functionality *in planta*. The hydrophobic motif is conserved across NFR5-type receptors in different plant species, suggesting a conserved signaling function. Indeed, the intracellular domain of NFR5 can be substituted with the corresponding domain of barley RLK10 and complement the *nfr5* Nod^- phenotype.

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HOW *LOTUS JAPONICUS* BLOCKS NODULATION WITH 'NOD FACTOR-COMPATIBLE' RHIZOBIA *SINORHIZOBIUM FREDII* HH103

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To establish the plant–rhizobia bacteria symbiotic interaction, rhizobia produce Nod factors in response to compatible plant flavonoids. Nod factors are perceived by specific plant receptors triggering a downstream signalling cascade and colonization of the root hair via infection thread (IT) formation. In addition, rhizobia deliver effector proteins into the host cell to alter host signalling or suppress host defence responses to facilitate colonization.

Sinorhizobium fredii HH103 is a broad-host-range bacterium but only can form non-colonized nodules with the model legume *Lotus japonicus*. Nevertheless, *L. japonicus* early symbiotic signalling genes are induced upon inoculation with HH103 suggesting that both host and rhizobia possess the flavonoids and Nod factors for a compatible interaction. Mutations in HH103 regulatory gene *nolR* can overcome this colonization block via IT formation[1]. NolR is a transcriptional repressor of *nod* genes and mutants in *nolR* exhibit increased Nod factor production. Similarly, mutations in an effector *nopC* enable IT colonization, suggesting that NopC is normally recognised by the plant immune system, activating plant defence and blocking nodulation[2].

The plant mechanisms underlying these mutant-dependent gains of nodulation are unknown. Despite exhibiting similar phenotypes, we hypothesize that the plant molecular mechanisms underlying colonization with the mutants differ. For example, the over-production of Nod factors might enable colonization with the *nolR* mutant. On the other hand, a reduction in plant defence might enable colonization with the *nopC* mutant. Here, we use an RNA-seq approach to investigate the *L. japonicus* responses responsible for the block to colonization in HH103 and compatibility with *nolR* and *nopC* mutants.

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CAPTURING THE UBIQUITINATION AND PROTEIN INTERACTION LANDSCAPE OF THE CENTRAL REGULATOR OF NODULATION TOO MUCH LOVE IN SOYBEAN

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Soybean is one of the most important plant-based protein sources for food and feed[1]. This legume acts as a natural nitrogen fertilizer by establishing a symbiotic relationship with nitrogen-fixing rhizobia in a process called nodulation[2,3]. As nodulation is energetically costly, the number of formed nodules is tightly regulated through a systemic pathway called autoregulation of nodulation (AON)[4,5]. The key component acting at the final stages of AON is TOO MUCH LOVE (TML), a root factor inhibiting nodule formation[4,6]. TML is a Kelch repeat F-box containing protein, hence predicted to mediate AON signaling via ubiquitination of still unknown target proteins. Despite long-lasting research efforts, the TML targets and interactors as well as the complete control mechanism remain elusive[6].

We have currently started the process of identifying interactors and ubiquitination targets of TML using ubiquitinomics, shotgun proteomics and interactomics. Novel TML signaling components will be functionally characterized to unravel their mode-of-action during AON. This project will advance our knowledge on the molecular mechanisms by which TML regulates nodulation in response to rhizobia, which will guide future research towards an optimized nodulation control to face increased food demands and minimize the use of nitrogen fertilizers.

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INVESTIGATING THE FUNCTION OF NOPC EFFECTOR DURING SYMBIOTIC NODULATION IN *LOTUS* SPP.

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The symbiotic nodulation process between legumes and rhizobia is regulated by multiple interactions that occur between them. Some type III effectors in rhizobia, similar to those found in pathogenic bacteria, have been known to suppress or promote symbiotic nodulation. However, the mechanisms by which host plants regulate nodule symbiosis when these effectors were injected into their cells are still unknown. Therefore, we have been investigating the function of NopC, a type III effector with a plant-specific motif produced by *Sinorhizobium fredii* HH103 and *Mesorhizobium loti* MAFF303099, using *Lotus* spp.

A mutant analysis in *S. fredii* HH103 showed that the NopC effector functions in an inhibitory manner towards *L. japonicus* Gifu, while having no effect on *L. burttii* under our experimental conditions, as compared to previous studies[1, 2, 3]. Interestingly, when the *NopC* gene from *S. fredii* HH103 was expressed in another rhizobium, *M. loti* MAFF303099, it promoted the forming of infection thread and nodule numbers in *L. japonicus* Gifu, suggesting that the same effector may have different functions in different rhizobium species.

In this congress, we will present the expression pattern of *NopC* and host cell localization of NopC effector during the symbiotic process. Additionally, we will present data on the NopC function of *M. loti* MAFF303099.

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ESTABLISHING NOVEL IMAGING APPROACHES TO STUDY SIGNALLING BETWEEN *LOTUS JAPONICUS* AND INTERACTING ROOT MICROBES AT A CELLULAR LEVEL

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Receptors present at the plasma membrane of root cells recognise microbe-associated molecular patterns (MAMPs) and initiate signalling pathways resulting in an appropriate plant response, i.e., immunity or symbiosis.

The components of the signalling cascades leading to either symbiosis or immunity have until now been studied by monitoring gene expression, or by phenotypic studies of knockout mutants. However, it has not yet been possible to visualise or quantify signalling dynamics in real-time in the root cells. Therefore, we have developed genetically encoded biosensors containing fluorescent proteins that can inform on the activation status (on/off, fast/slow) of the signalling cascades in symbiosis and immunity under different conditions. These biosensors enable us to dissect how the signalling takes place from different receptor variants.

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CONSTITUTIVE ACTIVATION OF A NUCLEAR-LOCALIZED CALCIUM CHANNEL COMPLEX IN *MEDICAGO TRUNCATULA*

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Ca^{2+} represents a ubiquitous and versatile secondary messenger and plays important roles in signaling pathways in eukaryotic organisms. Plant establishment of endosymbiotic associations with nitrogen fixing bacteria and nutrient acquiring arbuscular mycorrhizal fungi involves induction by microbial signals, a shared plant signaling pathway which has at its core, nuclear Ca^{2+} oscillations[1-3]. Such Ca^{2+} oscillations require a Ca^{2+} pump, and multiple channels (DMI1 and CNGC15s) that interact at the nuclear membrane[4-6]. However, how calcium oscillations are encoded by these channels is unknown.

We have isolated a gain-of-function mutation in the cation-selective channel DMI1 of *Medicago truncatula* (*M.truncatula*) that causes spontaneous nuclear Ca^{2+} oscillations, leading to symbiotic gene expression and spontaneous nodule formation.

The causative mutation sites at an interface between two RCK domains of DMI1, and additional mutation of hydrogen bonds- and salt-bridges-residues that stabalize this interface also cause spontaneous Ca^{2+} oscillations and nodulation in *M. truncatula*. This supports the hypothesis that structural changes associated with these RCK domains are intrinsic to the activation of the calcium channel complex.

We have been able to recapitulate symbiotic-like calcium oscillations in mammalian HEK293T cell lines by transferring gain-of-function mutations in DMI1, along with CNGC15s. The result indicated that these mutations alone are sufficient to coordinate calcium oscillatory behavior. It demonstrated that conformational changes in DMI1 and its complex with CNGC15 lead to activation of this nuclear-associated Ca^{2+} channel complex, which alone create the oscillatory behavior, providing insights into its native mode of induction. This symbiotic calcium channel complex is the novel mechanism for nuclear calcium release, as compared to what is defined from animal systems.

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NOD FACTOR SIGNALING CONTROLLED GENES IN *MEDICAGO TRUNCATULA* NODULES

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Legume nodule formation is induced by lipo-chito-oligosaccharides, known as Nod factors (NFs), that are secreted by rhizobium bacteria. NF induced transcriptional changes in roots have been intensively studied. Although it has been shown that NF receptors also accumulate in the apex of *Medicago truncatula* nodules, the transcriptional changes induced there by NF signaling have never been studied. Here, we studied this by using NF signaling mutant TE7, which is a weak allele of *IPD3,* and is blocked in rhizobial release. Nodule apices were isolated with laser microdissection and used for transcriptional analysis. By this we identified genes differentially expressed in TE7 nodule apices compared to wild type. To identify which of these genes depend on rhizobial release, we generated transcriptome data from apices of *VAMP721d&e RNAi* nodule. In these nodules rhizobial release is blocked by reduced activity of the symbiotic exocytosis pathway. We identified a subset of genes whose regulation of expression depends, directly or indirectly, on NF signaling and rhizobial release. Further, we compared the set of genes controlled by NF signaling in nodule apices with that controlled in root epidermis, and these were markedly different. Among the genes only regulated in nodules are genes required for rhizobial differentiation and intracellular accommodation, the expression of many of which depend on rhizobial release. *NIN* is induced by Nod factor signaling both in the root epidermis and in the nodule. By overexpression of *NIN* in TE7 and knock down of *NIN* in wildtype nodules we showed that NF signaling controlled rhizobial release depends on *NIN*.

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ROOT MERISTEM GROWTH FACTOR PEPTIDES AND THEIR EFFECTS ON ROOT DEVELOPMENT AND NODULATION IN SOYBEAN

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Nodulation involves complex molecular signalling pathways to control the physiological changes that are required to initiate and maintain nodules[1,2]. We have identified a peptide family in soybean (*Glycine max*), called Root Meristem Growth Factors (RGF), which are orthologous to Arabidopsis peptides that have essential roles in root meristem activity and immune response to regulate plant growth[3,4,5]. Within the gene family, we have functionally characterised a number of these using molecular techniques such as overexpression, synthetic peptide application and CRISPR. The results suggest they act in soybean nodule development and root growth, including regulating the maturity of the nodule. These novel findings improve our understanding of legume signalling and benefit future crop development programs to enhance biological nitrogen fixation and advance legumes to benefit health and environment towards agriculture sustainability.

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ANALYSIS OF THE *LYK* GENE CLUSTER IN TWO *MEDICAGO* GENOTYPES

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The legume-rhizobia symbiosis is one of the most well-characterized mutualistic interactions in nature which is playing an increasingly important role in sustainable agriculture. To establish the symbiotic interaction between the two partners, one of the key steps is the perception of rhizobium-secreted Nod factors (NFs) which has been shown to involve lysin motif receptor-like kinases (LysM-RLKs) [1,2].

In this study, we characterized a cluster of *LysM-RLK* genes, which is implicated in partner-specific recognition in two widely studied *Medicago* genotypes, A17 and R108. We found that the cluster exhibits a high degree of structural variability among *Medicago* genotypes, which includes recent recombination events within the cluster in both A17 and R108 and a transposon insertion in A17 [3]. Notably, we showed that the gene *LYK3* from R108 is not essential for nodulation, in contrast to A17, despite similar sequences and good nodulation expression profiles [3]. Using reverse genetics and *Sinorhizobium meliloti* mutants, we identified a newly-evolved *LysM-RLK*, specific to the R108 genotype, designated as *LYK2bis*, which allows R108 to nodulate with *S. meliloti* strains producing a specific NF decoration and is important for nodulation of R108 with some tested natural strains [4]. In addition, our study also included *LYK2*, *LYK5* and *LYK5bis:* although no essential roles were found for these genes, some evidence suggests that they may play accessory roles in nodulation [3].

This work has shown that recent evolution in the *LYK* gene cluster provides a source of variation for nodulation, which may represent an adaptive advantage to allow nodulation with a greater variety of strains and/or may improve robustness of signalling through genetic redundancy. Our study also shows the value of exploiting the genetic diversity of different genotypes/ecotypes for understanding the mechanisms of symbiotic signalling.

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MILDEW LOCUS O (MLO) PROTEINS ARE REQUIRED FOR RHIZOBIAL INFECTION IN *MEDICAGO TRUNCATULA*

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Mildew Locus O (MLO) proteins, named after the robust resistance of the barley *mlo* mutant to powdery mildew, are involved in many processes, including reproduction, thigmotropism, and biotrophic interactions with biotrophic fungi, which besides powdery mildew, also include the endophyte *Serindipita indica*[1], and arbuscular mycorrhizal fungi[2]. Their biochemical role remained a mystery for many years, but recent studies have revealed they act as calcium channels. We have identified a root-hair expressed MLO gene that when mutated strongly decreases rhizobial infection. A second root-hair expressed MLO gene, which encodes the ortholog of Arabidopsis NORTIA which is involved in reproduction, was found to be induced by rhizobia and Nod factors. A double mutant showed defects in infection and a decrease in nodule number. Transcript profiling, along with genetic and physiological studies, have provided insight into the underlying mechanism. Given that every biological process linked to MLOs so far involves contact with an external stimulus, we propose that MLOs are involved in sensing rhizobial attachment to direct infection thread growth.

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ROXR, A REDOX-SENSING REGULATOR OF *SINORHIZOBIUM MELILOTI*, IS CRUCIAL FOR SYMBIOTIC INFECTION OF *MEDICAGO TRUNCATULA* ROOTS

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Sinorhizobium meliloti is a soil bacterium that can establish nitrogen-fixing symbiosis with the legume *Medicago truncatula*. This interaction is initiated in the rhizosphere by a molecular dialog between the two partners. Subsequent plant root infection leads to the formation of a novel root organ, the nodule, where bacteria differentiate into bacteroids reducing the atmospheric nitrogen into ammonium. The regulation of *S. meliloti* intracellular redox state plays a major role in nodule development and functioning. Indeed, bacteria are exposed to reactive oxygen species (ROS) produced by the plant at different steps of the interaction, and several mutants of the antioxidant defense are affected in nodule development[1]. Moreover, ROS are known to play a role in redox signalling in particular by modifying the activity of redox-sensing transcriptional regulators (TRs). In bacteria, many of them belong to the MarR family. This work focused on a *S. meliloti* MarR-like TR, called RoxR, expressed during symbiosis[2]. Using both in vitro and in vivo approaches, we demonstrated that RoxR is a repressor that binds to its own promoter, and is inactivated by some oxidants (NaOCl, peroxides). The deletion of *roxR* did not significantly affects the growth rate of *S. meliloti* under normal conditions. However, using a redox-sensitive GFP, we showed that the mutant has an increased capacity to maintain intracellular redox homeostasis upon oxidant treatment. *In planta*, the deletion mutant was strongly affected in nodulation and nitrogen fixing capacities. An analysis of the expression of *M. truncatula* marker genes showed that the Δ*roxR* mutant induced an altered plant response at early infection stages. In addition, microscopic analyses of nodules showed that the Δ*roxR* mutant induced the formation of many non-invaded pseudonodules and rare nodules. Altogether, these results suggested that RoxR plays a key role in the redox regulation of symbiosis.

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THE PUTATIVE TYPE III EFFECTOR SKP48 OF *BRADYRHIZOBIUM* SP. DOA9 IS INVOLVED IN LEGUME NODULATION

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Bradyrhizobium sp. DOA9 can nodulate a wide spectrum of legumes; however, unlike other bradyrhizobia, DOA9 carries a symbiotic plasmid harboring type III secretion system (T3SS) with several effector (T3E) genes, one of which encodes a new putative type III effector—SkP48. Here, we demonstrated the pivotal roles of SkP48 from *Bradyrhizobium* sp. DOA9 in inhibiting nodulation of various *Vigna* species and *Crotalaria juncea* as well as suppressing nodulation efficiency of *Arachis hypogaea*. By contrast, the nodulation efficiency of a SkP48 mutant did not differ significantly with the DOA9 wild-type strain on *Macroptilium atropurpureum* and *Stylosanthes hamata*. An evolutionary analysis revealed that the SkP48 effector contains a shikimate kinase and a SUMO protease (C48 cysteine peptidase) domain. SkP48 is distinct from other effectors as previously identified in other bradyrhizobia and pathogenic bacteria. Our findings suggest that the new putative T3E, SkP48 is a key factor suppressing nodulation and nodule organogenesis in several legumes by activation of effector-triggered immunity through salicylic acid biosynthesis induction, which is deleterious to rhizobial infection. In addition, nodulation may be modulated by the function of defensins involved in jasmonic acid signalling in *V. radiata* SUT1.

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THE STRINGENT RESPONSE TRIGGERS THE EXPRESSION OF THE TYPE III SECRETION SYSTEM IN *BRADYRHIZOBIUM DIAZOEFFICIENS*

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Bradyrhizobium diazoefficiens is a soil bacterium that stablish a symbiosis with soybean plants. Soybean flavonoids induce the expression of the Type 3 Secretion System (T3SS) that injects effector proteins known as Nodulations Outer Proteins (Nops). These Nops are involved in the suppression of the host's defenses and host range expansion [1,2]. In *B. diazoefficiens*, the T3SS is activated by the flavonoid genistein through the transcriptional regulator TtsI, which also controls the expression of the Nops [3]. In some pathogens, the T3SS induction is affected by the second messenger (p)ppGpp. In response to starvation, bacteria trigger the stringent response (SR) and (p)ppGpp is synthesized. A *rsh* mutant of *B. diazoefficiens*, unable to synthesize (p)ppGpp, expresses less *ttsI* than the wild type strain in presence of genistein. As consequence, this mutant is not able to modulate the plant defense response, so its symbiotic performance is affected [4].

We hypothesize that *B. diazoefficiens* USDA 110 under C and N starvation, increases (p)ppGpp levels [4] and prepares rhizobia for symbiotic interaction through the regulation of the T3SS, stimulating the symbiotic interaction. We measured the transcript levels of *ttsI* by qRT-PCR in wild type and *rsh* mutant strains, in cultures with genistein, without induction and treated 1 or 24 hours with a C/N free mineral solution. We found that *ttsI* is overexpressed in nutritional stress condition than with genistein treatment in the wild type strain. In contrast, the *ttsI* expression in the *rsh* mutant was not affected in any condition. Other preliminary results from the T3SS's signalling cascade have shown that (p)ppGpp levels affect genes under *ttsI* regulation.

In order to find determinants linking SR and T3SS, we performed comparative proteomics. The analysis of the different metabolic conditions showed that the effects of stress and T3SS induction were achieved.

Furthermore, the host range was unaffected since the *rsh* mutant was capable of establish a symbiotic interaction with both soybean and mung bean plants.

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P 30

MtCel2, a new cellulase involved in the establishment of the *Medicago truncatula-Sinorhizobium meliloti* symbiosis

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In temperate legumes, the controlled entry of rhizobia into roots occurs via tubular structures, known as infection threads (ITs) whose construction is regulated by key plant transcription factors, such as *ERF Required for Nodulation 1* (*ERN1)* and *2* (*ERN2)*[1,2]. Comparative transcriptomic analysis of *M. truncatula* A17 (WT) and IT-defective (*ern1 & ern1ern2*) mutants revealed a novel rhizobium-induced cellulase encoding gene, designed *MtCel2,* whose expression is strongly down-regulated in the mutants. *MtCel2* encodes a secreted β-1,4-endoglucanase belonging to group B of the plant glycoside hydrolase 9 protein family[3], not yet characterized in a symbiotic context. We could confirm in *Nicotiana benthamiana* that *MtCel2* encodes an active endocellulase enzyme localized to the cell periphery. Expression analysis of a *MtCel2* promoter-GUS fusion in WT and mutant backgrounds revealed the precise spatiotemporal tissue-specific expression profile of *MtCel2* during rhizobia infection and nodule development, which we showed to be ERN1/ERN2 dependent. Transactivation assays in *N. benthamiana* further demonstrated the transcription activation of *pMtCel2* by ERN1/ERN2 regulators. Together, these data support that ERN1/ERN2 are upstream regulators of a cellulase-encoding gene, likely involved in cell wall modifications required for rhizobia colonization and nodule development. Functional strategies aimed at elucidating the relative contribution of *MtCel2* to nodulation will be presented.

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DECIPHERING THE TRANSCRIPTIONAL REGULATION BY THE CCAMK/ CYCLOPS COMPLEX DURING ROOT ENDOSYMBIOSES

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To overcome nutrient limitations, legumes engage in two main types of root endosymbioses with beneficial microbes called arbuscular mycorrhiza (AM) and nitrogen-fixing root nodule symbiosis (RNS) [1]. The establishment of both AM and RNS requires an overlapping set of signaling components (encoded by the so called “common symbiosis genes”) that include the nuclear localized calcium and calmodulin-dependent kinase CCaMK and its phosphorylation target Cyclops, a DNA-binding transcription factor [2]. The complex formed by CCaMK and Cyclops activates the transcription of distinct genes during AM and RNS development [2-5], however, it remains unclear how the transcriptional specificity of CCaMK/Cyclops is mechanistically achieved. To approach this issue, we study the specific role of Cyclops’ phosphorylation status and interactome in tuning the transcriptional activity of the complex.

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Sequence adaptation of Symbiosis Receptor-like Kinase (SymRK) enabling nitrogen-fixing root nodule development

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Root endosymbioses take place between plants and soil microbes and it involves the intracellular accommodation of fungi or bacteria. Arbuscular Mycorrhiza (AM), present in almost 80% of land plants, dates back to 450 million years ago. Root Nodule Symbiosis (RNS) emerged more recently, around 60 million years ago, and it is restricted to the orders of Fabales, Fagales, Cucurbitales and Rosales [1]. While the evolutionary origin differs among AM and RNS, they both require a set of conserved genes indispensable for symbiosis. It has been hypothesised that some of them have been co-opted during the evolution of RNS, including *Symbiosis Receptor-like Kinase* (*SymRK*) [2,3]. Based on experimental evidence we hypothesise that SymRK underwent super-functionalisation, acquiring new features necessary for the establishment of RNS, while maintaining its conserved function for AM [4,5]. In this project, we will explore the protein sequence diversity among SymRK orthologs, aiming to identify critical amino acid changes in *Lotus japonicus SymRK,* necessary for the establishment of RNS. We will study the mechanistic consequences of these adaptations at a cellular and molecular level. To achieve this goal, we will combine genome editing, diverse -omics and biochemical approaches.

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P 33

HOST RANGE GENETIC DETERMINANTS IN *MESORHIZOBIUM CICERI*

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Biserrula pelecinus is a herbaceous annual legume introduced into Australia from the Mediterranean basin in the 1990s to provide a hard-seeded drought-tolerant pasture. Australian soils lack native rhizobia capable of nodulating and fixing N_2 with *B. pelecinus*, leading to the introduction of inoculant strain *Mesorhizobium ciceri* WSM1497[1]. While WSM1497 effectively nodulates *B. pelecinus*, it has a narrow host range. In contrast, *M. ciceri* WSM1284[2] which fixes N_2 effectively with *B. pelecinus*, has a broad host range, being capable of nodulating other important pasture legumes (*e.g. Scorpiurus*, *Lotus*, *Ornithopus*). Understanding why differences in host specificity exist between these *Mesorhizobium* strains would facilitate better inoculation management practices in Australia. WSM1497 and WSM1284 genomes are highly similar (ANI 98.3%), consisting of a single chromosome with a tripartite symbiosis Integrative and Conjugative Element (or ICE^3)[3], and a *repABC* type plasmid (~0.5 Mb). Importantly, the strains do differ in their nodulation genes, with the WSM1284 ICE encoding 21 *nod* genes, while the WSM1497 ICE^3 has 14. Six of the additional WSM1284 genes appear to code for fucose biosynthesis (*noeL*, *nolK*, *noeJ*, *noeK*), transfer (*nodZ*) and acetylation (*nolL*). Both strains harbour multiple copies of *nodD* and *nodA* but their sequences diverge, indicating the NodDs may respond to different host flavonoids, and that Nod factors may vary in length and saturation of the acyl tail. Fourteen WSM1284 site-directed mutants have been constructed, targeting putative Nod factor transcriptional regulators (encoded by *nodD*) and biosynthesis (encoded by *nodA*, *nodZ* and *nolL*) genes, and have been evaluated *in planta* on several hosts. Additionally, to assess the role of the core genome in modulating host range, symbiosis genes encoded on ICEs have been transferred from both WSM1284 and WSM1497 into diverse non-symbiotic *Mesorhizobium* species[4]. Finally, potential host-range factors encoded on plasmids are being functionally assessed by curing the plasmid from both strains, using a plasmid incompatibility approach.

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P 34

NUTRIENT REGULATION OF LIPOCHITOOLIGOSACCHARIDE RECOGNITION IN PLANTS VIA *NSP1* AND *NSP2*

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Plants associate with mycorrhizal fungi and rhizobial bacteria for nutrient acquisition, and this relies on symbiosis signaling. Here we show that cereals can perceive lipochitooligosaccharides (LCOs), including Nod factors produced by nitrogen-fixing bacteria, for activation of symbiosis signaling. LCO perception in plants is activated by nutrient starvation, through transcriptional regulation of Nodulation Signaling Pathway (NSP)1 and NSP2. These transcription factors induce expression of an LCO receptor and act through the control of strigolactone biosynthesis and the karrikin-like receptor DWARF14-LIKE. This work has implications for sustainable productivity in cereals and legumes using mycorrhizal and rhizobial associations.

P 35

INVESTIGATING *LOTUS JAPONICUS* ROOT RESPONSE TO THE SEMI-COMPATIBLE RHIZOBIA *SINORHIZOBIUM FREDII* HH103 THROUGH SINGLE CELL RNA-SEQUENCING

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Lotus japonicus is a model plant species that has been extensively used to investigate the molecular mechanisms underlying symbiotic associations with nitrogen-fixing bacteria. Many transcriptome studies in *Lotus japonicus* have focused on interactions with fully compatible symbionts, whereas responses to non-adapted rhizobia are not well-characterized. By using a single cell RNA-sequencing (scRNA-seq) approach, we compared the root response of *Lotus japonicus* to the semi-compatible rhizobia *Sinorhizobium fredii* HH103 and to the fully compatible rhizobia *Mesorhizobium loti* R7A to identify genes that are potentially involved in differentiating between fully and semi-compatible symbionts.

Our results showed that known regulators involved in nodulation pathways as well as recently identified candidate genes with a role in the nodulation process [1] were strongly downregulated in *Lotus japonicus* root cells inoculated with *Sinorhizobium fredii* HH103. Additionally, we observed an enrichment in genes involved in cell wall modification, biosynthetic processes, defense response and redox regulation.

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P 36

OVEREXPRESSION OF SYMBIOTIC NF-YS SUPPRESSES NODULATION

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One of the key outputs of the nodulation signaling pathway is activation of the CCAAT-binding complex (CBC) which plays a positive role in infection and nodule formation. The CBC is a heterotrimer comprised of the DNA-binding NF-YA and histone-like NF-YB and NF-YC. One subunit, *NF-YB7* (*CBF3*) was shown have increased expression in *Medicago truncatula* roots upon colonization by arbuscular mycorrhizal fungi. We show that *NF-YB7* is induced by rhizobia and Nod factor treatments, and that loss of this gene reduces infection thread formation. To investigate the role of the CBC in infection, we overexpressed the three symbiotic subunits, *NF-YA1*, *NF-YB7* and *NF-YC2* (CBC-ox), in *Agrobacterium rhizogenes* induced hairy-roots. Unexpectedly, this resulted in almost complete repression of infection and nodule formation. Transcriptomic and genetic dissection of this phenomenon has provided insights into the role of the CBC in nodulation.

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A NOVEL INTERACTOR OF SYMBIOTIC RECEPTORS AFFECTS NODULATION AND IMMUNITY

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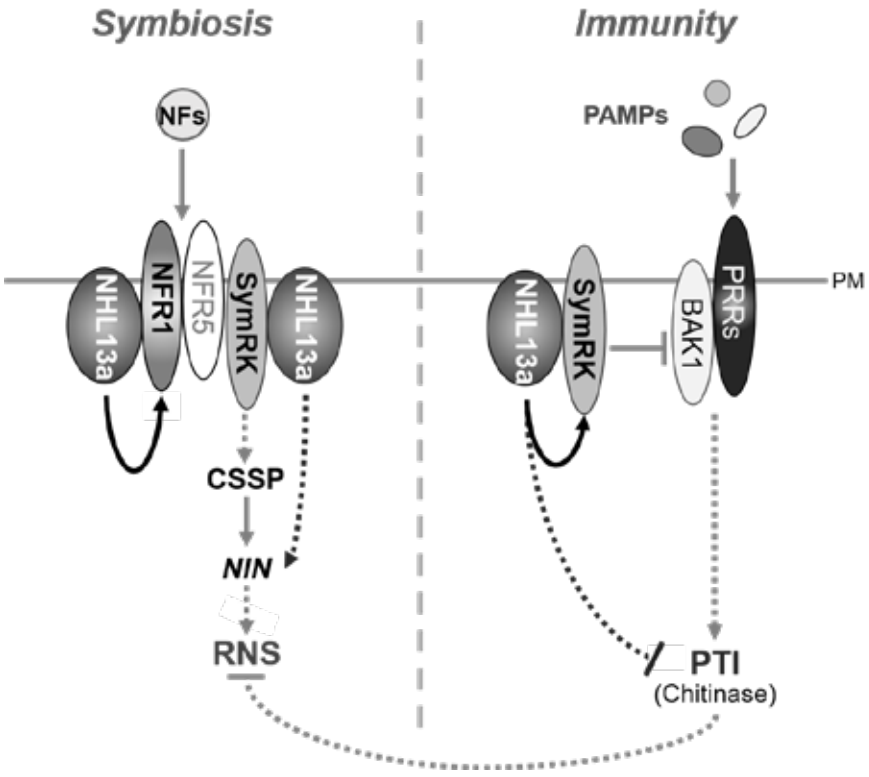
There is a plethora of cellular responses shared between symbiosis and immunity[1]. For instance, several symbiotic receptors are responsible for innate immunity[2,3]. Here, we identified NRD1/HIN1-Like protein 13a (NHL13a) as a novel player both in nodulation and in immunity in *Lotus japonicus*[4]. The membrane protein NHL13a interacted with NFR1 and SymRK, and positively influenced nodulation. The retroelement insertion in *NHL13a* did not alter arbuscular mycorrhiza, indicating its nodulation specific function. Upon PAMP treatment, the induction of *Chitinase* expression was higher in *nhl13a-1* than in wild type, displaying the involvement of *NHL13a* in the innate immune responses. Our findings provide an idea how plants coordinate the intertwined interactions between symbiosis and immunity.



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P 38

COMPETENCE FOR TRANSCELLULAR INFECTION IN THE ROOT CORTEX INVOLVES A POST-REPLICATIVE, CELL-CYCLE EXIT DECISION IN *MEDICAGO TRUNCATULA*.

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During root nodule symbiosis (RNS), cell-division activity is re-initiated and sustained in the root cortex to create a hospitable cellular niche. Such temporary and spatially confined site is required to render host cells compatible with the intracellular progression of rhizobia. Although it has been suggested that early infection events might involve a pre-mitotic cell-cycle arrest[1, 2], this process has not been dissected with cellular resolution. Here, we show that a dual-colour Medicago histone reporter robustly identifies cells with different mitotic or endoreduplication activities in the root cortex. By imaging deep root tissues, we found that a confined trajectory of cortical cells that are transcellularly passed by infection threads are in a stage of the cell-cycle that is distinct from directly adjacent cells. Distinctive features of infected cells include nuclear widening and large-scale chromatin rearrangements consistent with a cell-cycle exit prior to differentiation[3, 4]. Using a combination of fluorescent reporters demarcating cell-cycle phase progression, we confirmed that a reduced proliferation potential and modulating the G2/M transition, a process possibly controlled by the NF-YA1 transcription factor, mark the success of rhizobial delivery to nodule cells.

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IDENTIFICATION OF CIS-ACTING ELEMENTS INVOLVED IN THE TRANSCRIPTIONAL REGULATION OF THE CYSTEINE PROTEASE GENE *MTCP6* IN NODULE SENESCENCE OF *MEDICAGO TRUNCATULA*

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The leguminous plants associate with rhizobia to establish nitrogen-fixing symbiosis, leading to the formation of new root organs called nodules. However, lifespan of nodules is limited due to the senescence process, which ends with a complete degradation of bacteroids and host plant cells. The increased proteolytic activity is one of the hallmarks of nodule senescence [1]. In *Medicago truncatula*, a papain cysteine protease, *MtCP6*, is found to be involved in the nodule senescence initiation process, both under developmental and stress-induced nodule senescence [2]. In order to proceed to the analysis of the cis-regulatory elements present on the proximal promoter of *MtCP6*, progressive promoter deletions were constructed and analysed in vivo via the expression of the *MtCP6* promoter::*β glucuronidase* gene fusion in transgenic roots. In vivo, a minimal promoter sequence has been identified as sufficient for a specific spatiotemporal transcriptional activation in nodules. Furthermore, functional analysis has validated the cis-elements containing promoter fragment by gain- or loss-of-function. In this work a senescence nodule specific cis-regulatory element, thereafter, named NS-box, was uncovered as a key sequence in order to decipher the complex transcriptional regulatory network involved in the nodule senescence process.

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CHARACTERIZATION OF THE OmpR-TYPE REGULATORS REQUIRED FOR APPROPIATE BACTERIAL GROWTH IN *R. etli.*

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In bacteria, diverse biological important processes are controlled by the two-component systems (TCS). The most simplified scheme of the TCSs consists of regulatory pairs of one sensor histidine kinase (HK) and one response regulator (RR). Members of the OmpR/PhoB family of RRs are highly represented among bacterial genomes. They are involved in several regulatory pathways, such as metabolism, stress response, virulence, multidrug resistance, or host-microbe interactions. In *Rhizobium etli*, the soil-dwelling bacteria that establish a nitrogen-fixing symbiosis with the common bean *Phaseolus vulgaris*, 18 of its 68 RRs belong to the OmpR family. We are interested in the characterization of novel regulators and have focused on the OmpR-type regulators with less predictable functions. We obtained a set of individual mutants with a two-step recombination process to eliminate an *ompR* gene. Using this methodology, we recently described that the *R. etli* OmpR regulator RetPC57 is critical in developing the *R. etli* - common bean symbiosis[1]. However, we were not able to delete genes *RetCH3010* and *RetCH3968*. This result suggests that these genes are essential for bacterial survival or growth. In this work, we will discuss advances in the phenotypic characterization of conditional mutants in these genes. We will also show that these regulate the expression level of essential genes from pathways controlling cell growth, cellular morphology, or oxidative respiration in *R. etli* CFN42.

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Ethylene biosynthesis in legumes: a simple pathway with many actors

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The plant hormone ethylene is synthesized through the concerted action of the enzymes 1-aminocyclopropane 1-carboxylate synthase (ACS) and oxidase (ACO). These enzymes usually belong to relatively small gene families (5-6 members). In legumes, these families have not been characterized in detail and researchers usually rely on automatic genome annotation tools, which could lead to a misinterpretation of results. Furthermore, ethylene is a well-known inhibitor of legume-rhizobium symbiotic interactions for most legumes. However, the molecular mechanism underlying this regulation remains largely unknown. Identifying and defining the gene members of the ACS and ACO families is, therefore, necessary and a relevant piece of information for the symbiosis community.

In this work, we carried out a comprehensive analysis of the genes annotated as ACS and ACO in one of the latest *Medicago truncatula* genome versions (v5.1.8[1]). We retrieved the sequences and performed reciprocal BLAST and phylogenetic analysis to re-evaluate their annotation. We found two putative *ACS* genes which, instead, belong to the general aminotransferase family, and several others annotated as *ACOs* that belong to the 2-oxoglutarate and Fe(II)-dependent oxygenase superfamily. The analysis was also extended to other members of the legume family including soybean, peanut, *Lotus japonicus*, *Vigna unguiculata*, as well as non-legumes. Combined with expression profiling analysis, this work will establish the basis for further functional characterization studies.

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IMPACT OF RNASE III IN REGULATION BY SRNAS IN *SINORHIZOBIUM MELILOTI*

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Ribonucleases (RNases) are important elements of post-transcriptional regulatory networks that are poorly characterized in rhizobia. Prokaryotic endoribonucleases like RNase III, which is specific to double-stranded RNAs (dsRNA), are commonly involved in mRNA decay upon antisense interaction with regulatory small non-coding RNAs (sRNAs)[1,2]. We previously characterized *S. meliloti* RNase III (SmRNase III) biochemically and genetically [3]. Here, we analyzed *Sm*RNAse III-dependent alterations of the *S. meliloti* transcriptome under oxic and microoxic conditions, the latter mimicking the symbiotic environment within root nodules.

RNA-Seq revealed a strong impact of RNase III in the *S. meliloti* transcriptome as expected from the pleiotropic phenotype of the knock-out mutant. At least, 20% of annotated sRNAs in the *S. meliloti* genome are differentially expressed. We further correlated alterations in the steady-state levels of mRNAs with their corresponding antisense sRNAs (asRNAs). Among these targets, we found protein-coding genes involved in nitrogen metabolism and siderophore production pathways, which are being genetically analyzed.

This analysis anticipates a great impact of this endoribonulcease in the post-transcriptional RNA silencing of genes relevant to both the free-living and symbiotic rhizobial lifestyles.

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A BALANCED INTERACTION: IDENTIFICATION OF SOYBEAN NODULE AUTOREGULATION RECEPTOR KINASE TARGETS

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Soybean is the most commonly cultivated legume and an important crop for food, feed an oil production[1]. Most legumes, including soybean, can establish symbiotic interactions with rhizobia, which are nitrogen-fixing bacteria. The legume-rhizobia interaction results in formation of nodules on plant roots, where fixed nitrogen provided by bacteria is exchanged for carbohydrates. For an optimal balance between benefits and costs of this interaction, plants tightly regulate their nodule numbers. In soybean, rhizobia induce the expression of rhizobia-induced CLE (RIC) peptides which activate the autoregulation of nodulation (AON) pathway. These peptides are transported from root to shoot, where they are perceived by the Nodule Autoregulation Receptor Kinase (NARK)[2]. This results in secondary shoot-to-root signals to inhibit nodulation. In addition to the systemic AON pathway, NARK is involved in local signalling in the roots in response to nitrate availability. Nitrate-induced CLE (NIC) peptides, perceived by NARK, lead to inhibition of nodulation[2]. Limited knowledge is available about the downstream NARK pathway in roots and shoots, but signalling via phosphorylation is expected based on the function of NARK. To fill this research gap, we are currently performing phosphoproteomics in soybean roots and leaves in response to nitrate (10mM KNO_3) and rhizobia, respectively. In roots, eight time points between 5min and 24h after KNO_3 treatment are selected for phosphoproteomic analysis, based on expression induction of the *NIC* peptides. Similarly for leaves, expression analysis of *RIC* in roots and *miR2111* in leaves, a previously identified secondary signal[3], indicated a time frame of interest between 8h and 4 days post-inoculation. This time series phosphoproteomics will allow the elucidation of dynamic phosphorylation changes upon induction of NARK signalling, identifying putative phosphorylation targets of NARK.

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IDENTIFICATION OF SYSTEMIC EFFECTORS INVOLVED IN THE NITROGEN DEFICIT REGULATION OF NODULATION IN *MEDICAGO TRUNCATULA*

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Legume plants adapt their root system architecture to environmental conditions by modifying root growth and lateral root number. In the case of a mineral nitrogen deficit, they develop a new organ in response to rhizobium symbiotic bacteria: the nitrogen-fixing nodule. Local and systemic regulatory pathways coordinate root and nodule development depending on nitrogen availability, notably through signalling peptides perceived by Leucine-Rich Repeats Receptor Like Kinases. In the *Medicago truncatula* model legume, C-TERMINALLY ENCODED PEPTIDEs (CEPs) were reported as critical to determine root competence for symbiotic nodulation through the COMPACT ROOT ARCHITECTURE 2 (CRA2) receptor acting in shoots[1-4]. Effectors acting downstream of this CEP/CRA2 systemic pathway remain to be discovered either in shoots or in roots.

We used transcriptomic analyses to compare systemic responses of wild-type plants *versus cra2* mutants at an early stage after rhizobium inoculation, or in plants experiencing a nitrogen deficit or satiety. This allowed the identification of candidate genes acting downstream of the CEP/CRA2 pathway in shoots or in roots, either encoding transcription factors from different families or an enzyme involved in secondary metabolite production. Progress on the functional analysis of some of these CEP/CRA2 effectors will be reported.

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THE ALPHA-PROTEOBACTERIAL TRANS-ENCODED SMALL RNA MMGR: PROTEOMIC PROFILING REVEALS ROLE BEYOND POLYHYDROXYBUTYRATE REGULATION IN SINORHIZOBIUM MELILOTI

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In the N_2-fixing legume symbiont *S. meliloti*, *mmgR* encodes a 77-nt non-coding sRNA[1]. *mmgR* evolves from a common ancestor of the α-proteobacteria that diverged from the order of Rickettsiales, thus being so far the sRNA with the widest phylogenetic distribution described in this bacterial clade[2]. *mmgR* orthologs –grouped after a strong microsyntheny found with a conserved protein coding gene- share fully conserved structure and sequence traits along evolution. MmgR regulates the production of the major C- and reducing-power-storage polymer polyhydroxybutyrate (PHB) in *S. meliloti* cells growing under conditions of N starvation and C surplus. MmgR expression in *S. meliloti* sets a limit for PHB accumulation and modulates PHB-granule morphology[3]. The expression of MmgR is mainly regulated at the transcriptional level by the N and C metabolism master regulators NtrC and AniA, respectively[4, 5]. This regulation relies on a conserved dyadic motif located within the -35 and -10 boxes of the *mmgR* promoter, and results in activation of *mmgR* expression according to the C:N molar ratio in the growth medium upon exhaustion of the N source. MmgR is part of a regulatory loop that operates to maintain a proper structure and amount of PHB granules in *S. meliloti* through a fine-tuning of the intracellular levels of phasins and polymer, on the basis of the availability of N and C.

Here, we profiled the proteome of *S. meliloti* during growth in defined medium to dynamically follow the changes associated with modifying the intracellular activity of MmgR. We found that MmgR impacts on multiple targets beyond its known role in the regulation of PHB metabolism, which suggests its role as a regulatory hub associated with the transition phase to nutrient scarce conditions.

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COMPLEX REGULATORY NETWORKS GOVERN THE SYNTHESIS OF MOLECULAR SYMBIOTIC SIGNALS IN *SINORHIZOBIUM FREDII* HH103

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Rhizobia are soil bacteria able to establish a symbiotic nitrogen-fixing interaction with legumes. Rhizobia infect legume roots and intracellularly colonize new plant organs called nodules. Within these nodules rhizobia differentiate into bacteroids able to fix N_2 and provide combined nitrogen to the plant. The nodulation process is based on a complex molecular dialogue established between both partners that starts with the exudation of plant flavonoids that interact with the rhizobial transcriptional regulator NodD, which acts as the main inducer of the expression of bacterial genes related to symbiosis [1].

Sinorhizobium fredii HH103 is a broad host-range strain able to nodulate dozens of legumes including soybean, its natural host plant. In this strain, NodD1 is the main regulator that controls the production of symbiotic signals but also induces the expression of secondary transcriptional regulators such as TtsI, NodD2, and SyrM. In turns these proteins induce or repress the expression of genes coding for symbiotic signals. Additional global regulators, such as NolR or MucR1, also have a role in this symbiotic regulon. Inactivation of NolR, NodD2 or TtsI partially impairs nodulation with soybean but may extend nodulation to non-host plants such as *Lotus japonicus* and *Phaseolus vulgaris* [2].

In this work we analysed the transcriptional regulatory networks that govern the production of the symbiotic signals in *S. fredii* HH103, which can either block nodulation or allow this strain to nodulate a broad range of legumes.

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ANALYSIS OF THE DIFFERENTIAL ASSOCIATION BETWEEN ARGONAUTE PROTEINS AND SMALL RNAS IN THE REGULATION OF LEGUME-RHIZOBIA SYMBIOSIS

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The symbiotic relationship between Rhizobia and leguminous plants (Legume-Rhizobia Symbiosis or LRS) is a process that has been extensively studied to identify the components that participate in its regulation. One of these components is the regulation induced by small RNAs (sRNAs), which guide gene silencing to modulate numerous processes in most eukaryotes; particularly, the plant-microorganism interactions, including the different stages of the LRS. Argonaute (AGO) family proteins are crucial for the functioning and action mechanisms of sRNAs, being an essential part of the RNA Induced Silencing Complex. Previously, the role of several differentially accumulated sRNAs in LRS had been identified and evaluated [1], however, little has been studied about their differential association with AGO proteins in this context. Furthermore, the preferential AGO loading of sRNAs seems to have a significant impact on gene silencing and potentially influence LRS. For example, mechanisms like “microRNA sequestration” [2] make AGO1 and AGO10 compete for miR165/166 association [3] and therefore impair the regulation of transcripts involved in root development [4,5].

In the present work, we selected the model legume *Phaseolus vulgaris* in interaction with *Rhizobium etli* to analyze the differential and preferential loading of sRNAs in AGO1 and AGO10. We observed the AGO loading profile of miR166 when comparing 5dpi inoculated plants with non-inoculated controls, giving rise to further study the functional role of this microRNA in the LRS context.

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CARBON CATABOLITE REPRESSION AND CARBON UTILIZATION REGULATION IN RHIZOBIA

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Nitrogen-fixing rhizobia bacteria are equipped with global regulatory mechanisms that enable them to selectively assimilate a preferred carbon source from a mixture of available sources, a process referred to as carbon catabolite repression (CCR). This ability to optimize catabolism via CCR can enhance the competitiveness of rhizobia in their natural environment which has implications for their effectiveness in nitrogen fixation and potential applications in agricultural research.

We focus on exploring the molecular mechanisms involved in CCR and its impact on the expression of genes related to carbon utilization and nitrogen fixation in rhizobia. For instance, in *Rhizobium leguminosarum* bv. *viciae* 3841, the uptake of other carbon sources is repressed when the preferred carbon source succinate, a dicarboxylate, is present. We were able to conduct a high-throughput screen to identify mutants with defective CCR by combining a luminescence-based reporter[1] and a transposon-based genome-wide random mutagenesis library[2]. For this, a plasmid-based bacterial *lux* fusion reporter was constructed to measure the promoter activity of the gene encoding the *myo*-inositol transporter (*intA*), which usually is repressed in presence of succinate. Conjugating the reporter-plasmid into the mutant library thus allowed us to find CCR-suppressor mutants by scoring the luminescence of individual colonies plated on media containing both *myo*-inositol and succinate. Subsequently, insertion-sites of the transposon within selected mutants were mapped via PCR.

Most mutations identified were found in one of the three genes encoding the dicarboxylate transport system (*dctA, dctB,* and *dctD*). We hypothesise that CCR in rhizobia is directly regulated by one or more of these proteins acting as global regulators, potentially by triggering inducer exclusion when a preferred carbon source is present.

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ROLE OF THE *BRADYRHIZOBIUM DIAZOEFFICIENS* CLPAP$_1$S$_1$ PROTEOLYTIC SYSTEM IN THE ABIOTIC STRESSES RESPONSE AND IN SYMBIOSIS

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Rhizobia-legume symbiotic interaction implies severe physiological changes of bacteria from the free-living state in the soil to the oxygen-limiting environment of plant root nodules. Moreover, environmental stresses are limiting factors for an effective nitrogen-fixing symbiosis. Clp-type chaperone-proteases are conserved energy-dependent proteolytic systems that degrade unfolded or misfolded proteins, as well as specific substrates[1]. They consist of an AAA+ ATPase-type chaperone, which recognizes and denatures the substrate, and a protease cylinder. Specific adapters also modulate their proteolytic activity. The function of Clp-type proteins in the response to abiotic stresses is well recognized, though a limited knowledge is available in the context of rhizobia, both in free-living and symbiotic states[2].

Here, we studied the role of the ClpAP$_1$S$_1$ chaperone-protease system of the soybean endosymbiont *Bradyrhizobium diazoefficiens*, which is involved in the proteolytic control of FixK$_2$, a key regulator for the microoxic metabolism of this bacterium[3]. To this end, strains deficient in the chaperone ClpA and its adaptor ClpS$_1$, as well as a ClpP$_1$ protease overexpressing strain were characterized. While the ClpA chaperone was needed for tolerance to heat shock and acid pH, *clpS$_1$* mutation increased resistance to saline stress and the entire ClpAP$_1$S$_1$ system resulted to be involved in the tolerance to alkaline pH. Inoculation of soybean plants with single or mixed-inoculum (parental:mutant) at different ratios showed that both *clpA*- and *clpS$_1$*-mutants were affected in the infection and nodulation processes as well as in nodule occupancy. These results point that both ClpA and ClpS$_1$ proteins might play a role at some stage of nodule colonization, which is currently under investigation.

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ROLE OF THE CCKA-CHPT-DIVL COMPLEX IN THE PHOSPHORYLATION OF THE MASTER REGULATOR CTRA DURING THE CELL CYCLE AND NITROGEN-FIXING SYMBIOSIS IN *SINORHIZOBIUM MELILOTI*

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Sinorhizobium meliloti is an alphaproteobacterium which is able to live free in the soil or in symbiosis with legumes. During symbiosis, bacteria fix atmospheric nitrogen within symbiotic organs, called nodules, where they undergo extreme cell differentiation into bacteroid. Bacteroids are characterized by genome endoreduplication, cell enlargement and high membrane permeability. The transcription factor CtrA has been shown to be the master regulator of the cell cycle and the transition from a free to a symbiotic lifestyle is accompanied by a gradual disappearance of CtrA during the differentiation of bacteroids, suggesting that the (de-) regulation of the cell cycle by CtrA is a crucial point for the establishment of the symbiosis. In the alphaproteobacterium *Caulobacter crescentus*, a bacterium related to *S. meliloti*, cell differentiation is also closely related to the cell cycle via the activity of the master regulator CtrA. CtrA has been shown to be activated by phosphorylation via a phosphorelay system consisting of the two histidine kinases DivL and CckA and the histidine phosphotransferase ChpT. Orthologs of these different regulators are present in *S. meliloti*, suggesting a conservation of this module in the regulation of CtrA in this bacterium. The objective of this work is to study the functions of the CckA-ChpT-DivL complex and its impact on CtrA in *S. meliloti* in free and symbiotic life. We first confirmed the essentiality of *divL* in *S. meliloti* by transduction of a deletion cassette. The study of a DivL-depletion strain allowed us to demonstrate that DivL is essential for the proper functioning of the cell cycle and that it is involved in the regulation of CtrA. A translational fusion with the fluorescent protein YFP showed that DivL is localized to a single pole. We also purified the phosphorelais proteins and reconstructed a part of the phosphorylation cascade in-vitro. Finally, the DivL-depletion strain is not able to perform an efficient symbiotic relationship with *Medicago sativa* under the tested conditions.

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ROLE OF FLAVONOIDS AND ISOFLAVONOIDS IN STRESS RESPONSE AND NODULATION IN THE MODEL LEGUME *LOTUS JAPONICUS*

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It is well established that flavonoids and isoflavonoids are important secondary metabolites which have roles in plant growth and development, defense against biotic and abiotic stres and establishment of the interaction between legumes and rhizobia [1,2,3]. Isoflavonoids, in particular, are mostly produced by legume species. However, little is known on the reason why this group of plants produces these particular compounds, how they do that and if there is any specific relevance of isoflavonoids for the plants. There are evidences that isoflavonoids are also involved in the systemic AON regulation of nodule number[4]. Different (iso)flavonoids can also be used by the host in order to exert and inhibitory effect on incompatible rhizobia[4]. Several works from our groups indicate that *L. japonicus* uses a peculiar strategy which is the accumulation of the isoflavonoid vestitol in response to different kinds of abiotic stress[5,6]. This is why we are searching for TF that may regulate differentially flavonoids and isoflavonoids biosynthetic pathways. Co-expression networks were built in our laboratory that established the existence of interconnections between different MYB transcriptional factors (TFs) genes and genes related to flavonoid and isoflavonoid biosynthetic in *Lotus japonicus* [2]. Homozygous mutant plants affected in the TFs genes identified were selected from the progeny of different lines containing the LORE1 transposon inserts. The results show several differences between wild type and MYB mutant plants in growth patterns and abiotic stress responses under symbiotic and non-symbiotic conditions.. Possibles changes in (iso)flavonoid levels as well as levels of expression of the genes involved in the biosynthesis of flavonoids and isoflavonoids are currently being studied in these mutant plants.

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GENETIC DETERMINANTS OF AMMONIUM EXCRETION IN *NIFL* MUTANTS OF *AZOTOBACTER VINELANDII*

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Nitrogen fixation in *A. vinelandii* is regulated by the NifL-NifA two-component system, where NifL acts as an anti-activator that tightly controls the activity of the Mo-nitrogenase -specific transcriptional activator NifA in response to redox, nitrogen, and carbon status. While several studies reported that mutations in *A. vinelandii nifL* resulted in the deregulation of nitrogenase expression and the release of large quantities of ammonium, knowledge about the specific determinants for this ammonium-excreting phenotype is lacking. In this work, we report that only specific disruptions of *nifL* lead to large quantities of ammonium accumulated in liquid culture (~12 mM). The ammonium excretion phenotype is associated solely with deletions of NifL domains combined with the insertion of a promoter sequence in the orientation opposite to that of *nifLA* transcription. We further demonstrated that the strength of the inserted promoter could influence the amounts of ammonium excreted by affecting *rnf1* gene expression as an additional requirement for ammonium excretion. These ammonium-excreting *nifL* mutants were able to significantly simulate the transfer of fixed nitrogen to rice compared with the wild-type strain. As an approach to tailor association between ammonium-excreting bacteria and specific crops, we have engineered novel strains in which the expression of mutant *nifLA* operons is driven by endogenous inducible promoters and are testing their ability to excrete ammonium in response to specific carbon sources. In addition, we have generated a structural model of NifL and have utilised a combination of biochemical and structural approaches to investigate the conformational dynamics of NifL in response to oxygen and energy signalling. These studies will guide the generation of efficient associative ammonium-excreting bacteria able to transfer fixed nitrogen to important crops.

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Sinorhizobium fredii HH103 SURFACE MOTILITY IS INDUCED BY FLAVONOIDS AND THE NodD1 AND TtsI BACTERIAL REGULATORY PROTEINS

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Bacteria can move on surfaces to colonize new environments and get more resources. Rhizobia are soil proteobacteria able to establish a symbiotic nitrogen-fixing interaction with legumes relying on a complex signal interchange between both partners. The interaction between the flavonoids exuded by legumes and the bacterial transcriptional activator NodD regulates the transcription of different rhizobial genes (the so-called nod regulon) and, with the participation of additional bacterial regulatory proteins (such as TtsI, MucR or NolR), influence the production of different rhizobial molecular signals. In *S. fredii* HH103, a broad host-range rhizobia, the nod-gene inducer flavonoid genistein and NodD1 trigger the production of Nod Factors as well as the type 3 secretion system (T3SS) assembly and the subsequent effector proteins secretion, but repress the exopolysaccharide production and biofilm formation [1,2]. We report that genistein promotes a surface translocation which involves both flagella-dependent and independent mechanisms, but not affects swimming motility. This surface motility is regulated in a flavonoid-NodD1-TtsI-dependent manner, relies on the assembly of the symbiotic T3SS, and involves the participation of additional modulators of the nod regulon (NolR and MucR1) [3]. To the best of our knowledge, our investigations show for the first time in a rhizobial strain that the inducer flavonoids can activate both T3SS synthesis and surface motility.

We will also show that the *S. fredii* HH103 SFHH103_00346-SFHH103_00348 genes, whose expression is driven by a previously unknown and not fully conserved *tts* box (a rhizobial promoter sequence where TtsI, the main regulator of T3SS, binds and activates the gene expression), is involved in genistein-induced surface motility.

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P 54

INVESTIGATING RHIZOBIA INDUCED MEMBRANE INVAGINATIONS IN AN *IN VITRO* MODEL SYSTEM

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A key element of the Root Nodule Symbiosis (RNS) is the intracellular infection of the host plant by the bacterial symbionts. This is achieved by the host-controlled formation of a polarly growing membrane tube, the infection thread (IT), from the infection chamber (IC) of the curled root hair. The mechanism behind the transition from an IC to polar IT growth, however, remains unclear. We identified the membrane bound LECTIN DOMAIN PROTEIN 1 (LDP1) in *Medicago truncatula* to be required for successful infection. LDP1 binds to an extracellular rhizobial matrix compound and is located at the IC and IT membrane during infection. Our findings suggest that the unipolar association of the LDP1 lectin domain with *Sinorhizobium meliloti* induces membrane invaginations at the IC, which in turn may trigger the onset of polar membrane growth. We will report on a high-throughput *in vitro* system that enables us to study membrane topologies induced/stabilized by different symbiotic proteins and engineered derivatives. With this, we aim to mechanistically understand rhizobium-induced membrane invaginations and infection thread maintenance in full detail.

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PLEIOTROPIC EFFECTS OF PHAR REGULATOR IN *BRADYRHIZOBIUM DIAZOEFFICIENS* METABOLISM

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Bradyrhizobium diazoefficiens can live inside soybean root nodules and in free-living conditions. In both states, when oxygen levels decrease, cells adjust their protein pools by gene transcription modulation. PhaR encodes a transcription factor annotated as PHA (polyhydroxyalkanoate) accumulation regulator. In a previous work, we found that mutation of *phaR* impaired PHB accumulation in free-living cells and in bacteroids, and also promotes higher plant shoot dry weight and competitiveness for nodulation with soybeans[1].

In this work, we found that under microoxic conditions (0.5% O_2 in the gas phase), PhaR not only controls the PHA cycle but also acts as a global regulator of excess carbon allocation by controlling the expression of a large set of genes, including *fixK*$_2$ and *nifA* genes, both encoding key transcription factors for microoxic and symbiotic metabolism in *B. diazoefficiens*[2]. Indeed, we expanded PhaR function by a multi-pronged approach that includes analysis of the effects of *phaR* mutation at transcriptional and protein levels, and of direct control of targets by PhaR determined by EMSA assays. We also were able to identify PhaR and other proteins associated with *B. diazoefficiens* PHA granules which indicates that PhaR acts as at both at gene regulation and at PHA biosynthesis levels.

Altogether, these findings confirm our hypothesis that PhaR is a regulator with global and pleiotropic effects on carbon flux which is currently under further investigation.

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A SHOOT DERIVED MIRNA ORCHESTRATES N-DEPENDENT ROOT ORGAN FORMATION

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In a natural soil environment, nitrogen availability is often limiting and not uniformly distributed. To ensure sufficient nitrogen supply, many seed plants adapt their root system architecture in response to nitrogen availability in the substrate, a process known as foraging response[1]. Nitrogen-dependent root system architectural adaptations are shoot dependent[2] and integrate the systemic nitrogen status of the plant. Similarly, the number of root symbiotic nodules in legumes is regulated according to nitrogen availability in a systemic process called Autoregulation of Nodulation (AON)[3]. We show that a shoot derived microRNA and its root expressed target regulate both bacterial endosymbiosis and N-dependent root system adaptation. The microRNA acts as a mobile shoot signal translocating to the root in a nitrogen homeostasis-dependent manner to control lateral root initiation. Intriguingly, the microRNA-target node is functionally conserved across plant lineages including the asymbiotic ruderal *Arabidopsis thaliana* and the legume model *Lotus japonicus*, identifying it as an essential, evolutionarily stable factor in shoot dependent adaptation of root organ formation in response to nitrate availability in plants of divergent lifestyles.

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DECIPHERING THE REGULATORY MECHANISMS THAT CONTROL SURFACE MOTILITY IN *SINORHIZOBIUM MELILOTI*: THE ROLE OF DNAJ

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Bacterial surface motility is a complex microbial trait that contributes to host colonization[1]. However, the knowledge about regulatory mechanisms that control surface translocation in rhizobia and their role in the establishment of symbiosis with legumes is still limited. Recently, 2-tridecanone (2-TDC) was identified as an infochemical in bacteria that hampers microbial colonization of plants[2]. In *Sinorhizobium meliloti*, 2-TDC promotes a mode of surface motility that is mostly independent of flagella[2]. To understand the mechanism of action of 2-TDC in *S. meliloti* and unveil genes putatively involved in plant colonization, Tn*5* transposants derived from a flagellaless strain that were impaired in 2-TDC-induced surface spreading were isolated and genetically characterized. In one of the mutants, the gene coding for the chaperone DnaJ was inactivated. Characterization of this transposant and newly obtained flagella-minus and flagella-plus *dnaJ* deletion mutants revealed that DnaJ is essential for surface translocation while it plays a minor role in swimming motility. DnaJ loss-of-function reduces stress tolerance in *S. meliloti* and hinders the establishment of efficient symbiosis by affecting nodule formation efficiency, cellular infection and nitrogen fixation[3]. Our work highlights the role of DnaJ in the free-living and symbiotic lifestyles of *S. meliloti*, and validates our approach for the discovery of bacterial genes relevant for plant colonization.

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A MULTILAYERED NETWORK INTEGRATING NITROGEN, CARBON, AND ENERGY METABOLISM IN *AZOSPIRILLUM BRASILENSE*

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PII proteins are well known for their role in regulating nitrogen metabolism in prokaryotes. However, there is growing evidence that PII may play a more significant role in bacterial metabolism than previously thought because of its ability to bind to three different effectors - ATP, ADP, and 2-oxoglutarate - and to interact directly with target proteins in response to these effectors. Using ligand-fishing assays and mass spectrometry, we identified 40 additional proteins in *Azospirillum brasilense* that are potentially regulated by PII and are involved in various metabolic pathways, including nitrogen metabolism, fatty acid metabolism, cofactor biosynthesis, nucleic acid metabolism, transcription, and signal transduction. The interactions were validated primarily by *in vitro* protein interaction assays and analyses of metabolic changes in response to nitrogen levels and the presence or absence of PII. The results indicate that PII is involved in the regulation of the enzyme acetyl-CoA carboxylase, leading to changes in fatty acid synthesis, regulation of the enzyme NAD synthetase, which is involved in the NAD^+ synthesis pathway, and changes in c-di-GMP metabolism, demonstrating that PII plays a pleiotropic role in bacterial metabolism. Our results demonstrate the critical role of PII in the integration of nitrogen, carbon, and energy metabolism in bacteria.

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IMPLEMENTATION OF GENETICALLY-ENCODED FLUORESCENT PROBES TO STUDY THE COORDINATION OF CARBON/NITROGEN METABOLISMS IN *NOSTOC SP* PCC7120

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Diazotrophic cyanobacteria fix both atmospheric carbon (C) and nitrogen (N) into biomass and are thus placed at a strategic intersection between the biogeochemical cycles of these two elements in nature. In these organisms, C and N assimilation pathways are tightly coupled, and their coordination is necessary to avoid metabolic inefficiencies and ensure optimal growth. Understanding how this functional coordination works is key to develop robust biotechnological approaches to use these organisms as biofertilizers in agriculture. In our model, *Nostoc sp* PCC7120, N and C assimilation are split into two distinct cell type, namely heterocyst and vegetative cell. To provide a favourable environment for N_2 fixation, heterocysts dismantle photosystem II and stop C fixation, thus becoming dependent on vegetative cells for chemical energy (ATP), reducing power (NADH) needed for N_2 fixation and C skeletons (2-oxoglutarate, 2-OG) required for N assimilation. On the other hand, heterocysts provide to vegetative cells fixed N to sustain C metabolism.

To investigate this functional coordination, we implemented genetically-encoded fluorescence probes to measure *in vivo* fluxes between the two metabolisms. Target probes are expressed under the control of cell specific promoters and allow specific quantification of cellular pH (cp-YFP, pH-GFP), redox state (GRX1-roGFP2, HyPer7), ATP (Ateam), NADH (Peredox-mCherry) and the key C/N metabolic hub 2-OG (PII-TC3). The exploitation of such ratiometric probes will allow to follow short time-scale oscillations of cellular environment and metabolic resources that affect the coordination between C and N metabolisms and investigate such variations in response to external inputs (e.g. light and CO_2 availability).

P 60

PROTEOMICS-BASED APPROACH TO IDENTIFY NOVEL PLAYERS INVOLVED IN AUTOREGULATION OF NODULATION IN *MEDICAGO TRUNCATULA*

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Legumes establish a symbiotic relationship with nitrogen-fixing rhizobia via nodulation in which atmospheric nitrogen is converted into assimilable plant nutrients. As nodulation is energetically costly, the nodule number is tightly controlled through the process of systemic autoregulation of nodulation (AON). In *Medicago truncatula* the AON pathway is initiated in response to rhizobia (*S. meliloti*) by upregulating the expression of CLAVATA3/Embryo Surrounding Region (CLE) peptides, *MtCLE12* and *MtCLE13*. The Kelch repeat-containing F-box proteins TOO MUCH LOVE 1 and 2 (MtTML) are the key factors in the final stage of AON potentially functioning by ubiquitination and proteasome-mediated degradation of its targets. To identify the potential targets of MtTML1/2, as well as novel components of the AON pathway, we used shotgun proteomics in the *M. truncatula* composite plants with transgenic roots ectopically expressing *MtCLE13* or downregulating Mt*TML1/2.* Several proteins with different functions were detected as differentially accumulating in samples with overexpressed *MtCLE13* and downregulated *TML1/2* when compared to the control (*35S::GUS*). The involvement of identified proteins in nodulation was further validated by testing the expression profile of the corresponding genes in the *M*. *truncatula* roots at different time points after the inoculation with *S. meliloti.* The progress in this research will be discussed.

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CELL WALL MODIFICATIONS AT THE SYMBIOTIC INTERFACE

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The root nodule symbiosis with its global impact on nitrogen fertilization of soils is characterized by an intracellular colonization of legume roots by rhizobia. To establish rhizobial symbiosis, rhizobia have to pass successively through several root cortex layers and later nodular cell layers. Throughout the transcellular passage, rhizobia must overcome cell wall barriers. Recently, we demonstrated that the coordinated function between symbiont-specific pectin methyl esterases (SyPMEs) and nodule pectin lyase (NPL) mediates local cell wall pectin modification, which is a necessary process that enables successful intracellular colonization by rhizobia[1]. Here, we will also present additional data demonstrating that pectin modification is also a key aspect in maintaining the function of infection droplets to ensure the successful release of rhizobia.

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REGULATION OF CENTRAL CARBON METABOLISM AND CARBON STORAGE BY THE PTS^{NTR} IN *RHIZOBIUM LEGUMINOSARUM*

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Careful coordination of intracellular carbon and nitrogen levels is crucial for the optimal growth of rhizobia and for the maintenance of efficient symbiosis with their legume host. In rhizobia and other α-proteobacteria, the balance of these macronutrients is controlled by the Phosphotransferase System (PTS^{Ntr}) at the post-translational level by protein-protein interactions. This system comprises an initial sensor protein PtsP, a phosphotransfer protein NPr, and two output regulatory proteins, PtsN and ManX[1,2]. PtsP autophosphorylates in the presence of phosphoenolpyruvate (PEP), but this is inhibited by the allosteric binding of glutamine, which signals nitrogen-rich conditions to its N-terminal GAF sensing domain. In the absence of glutamine under nitrogen limitation, autophosphorylated PtsP transfers the phosphate to NPr, which is then transferred to PtsN and ManX. Depending on their phosphorylation states, PtsN and ManX regulate downstream cellular functions. Unphosphorylated ManX activates the tricarboxylic acid (TCA) cycle, while phosphorylated PtsN activates ABC transporters and interacts with the two-component regulatory system ChvI/ChvG controlling exopolysaccharide (EPS) secretion[1].

In this project, our aims are to elucidate the molecular mechanisms and potential effectors of PTS^{Ntr} responsible for the regulation of carbon storage. We hypothesise that under nitrogen-poor conditions, excess carbon is allocated into different carbon polymers, such as the internal polymers polyhydroxybutyrate (PHB) and glycogen or the external surface polymer EPS[3], and these dynamic metabolic changes are likely to be mediated through PTS^{Ntr}. To test this, we employed various biochemical techniques to measure TCA cycle activities and quantify intracellular and extracellular polymer production. Our results reveal that PTS^{Ntr} affects the accumulation of carbon polymers by acting on TCA cycle dehydrogenase enzymes, resulting in increased carbon flux from the TCA cycle into storage.

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VISUALIZING HETEROGENEOUS EXPRESSION PATTERN OF NITROGEN FIXATION BY THE REPORTER SYSTEM IN UNICELLULAR DIAZOTROPHIC CYANOBACTERIUM *CROCOSPHAERA SUBTROPICA* ATCC 51142

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Diazotrophic cyanobacteria have developed two strategies to protect nitrogenase from oxygen evolved via photosynthesis, as spatial separation with heterocyst and temporal regulation by circadian clock. In addition, we have reported heterogeneous nitrogen fixation (HNF) in unicellular diazotrophic cyanobacteria *Crocosphaera*, which are abundant fixers in oligotrophic oceans [1]. Maximum 60 % of cells showed nitrogen fixation activity despite almost homogeneous carbon fixation. This cell-to-cell heterogeneity of nitrogen fixation allows to expand their ecological niche [2]. To track the mechanism of HNF at transcriptional level, we attempted to establish the reporter system based on the expression of fluorescence protein fused with a promoter of *nif* gene cluster in *C. subtropica* ATCC 51142. The genome of *C. subtropica* ATCC 51142 possesses an ortholog of transcriptional regulator of *nif* gene cluster CnfR as well as similar CnfR consensus sequence of *nifP* with it from a filamentous cyanobacterium *Leptolyngbya boryana* dg5 [3]. Therefore, we hypothesized that *C. subtropica* ATCC 51142 uses same transcriptional regulation with *Leptolyngbya* and obtained the transformant harbouring the promoter of *nifP* fused with teal fluorescent protein (TFP). As consistent with our previous report, fluorescence was detected from part of cells in a culture during night time. Furthermore, we observed different patterns of fluorescence-detected cell population according to availability of inorganic nitrogen source. Thus, our results implied the insight that HNF occurs at the transcriptional level as well.

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NON-IONIC OSMOTIC STRESS INDUCES THE BIOSYNTHESIS OF NODULATION FACTORS AND AFFECTS OTHER SYMBIOTIC TRAITS IN *SINORHIZOBIUM FREDII* HH103

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Rhizobia are soil proteobacteria that establish a symbiotic relationship with host legumes. This interaction requires a complex molecular dialogue in which bacterial NodD proteins play a very important role activating the expression of symbiotic genes, when in the presence of appropriate flavonoids. Among rhizobial symbiotic genes, nod genes are responsible for the synthesis of signals molecules called nodulation factors (NF), that are crucial for establishment of symbiosis. Interestingly, in *Rhizobium tropici* CIAT899, besides flavonoids, another factors, like osmotic or saline stress, activate *nod* gene expression [1,2].

We have investigated whether osmotic stress has also an effect on traits related to symbiosis in another rhizobial strain, *Sinorhizobium fredii* HH103 [3]. In this communication we show that the presence of mannitol 400 mM affects the expression of hundreds of genes, as assessed by RNAseq, and affects different bacterial traits such as motility, production of exopolysaccharide, NF, acyl homoserine lactones and indole acetic acid (IAA) [4].

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CHARACTERIZATION OF SUBTILASE GENES AS NOVEL REGULATORS IN THE LEGUME-RHIZOBIAL SYMBIOSIS

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Legume plants have evolved the ability to associate with beneficial bacteria called rhizobia, leading to the formation of root nodules, in which rhizobia propagate and fix N_2. The establishment of the symbiosis requires the successful intracellular infection of host cells. Here, we identified two genes encoding putative symbiotic, apoplastic proteases (SAP1 and SAP2) in *Medicago truncatula*, that may act as potential regulators of nodulation. First, we determined their spatiotemporal expression patterns using promoter-GUS assays. Our results indicate that *SAP1* promoter activity was specifically associated with infection. Here, *SAP1* expression is specifically induced in root hairs and cortical cells harboring infection threads. By contrast, *SAP2* expression is mainly restricted to nodule and lateral root primordia. To test the genetic impact of these proteases on infection thread progression and nodulation, we expressed a genetically encoded inhibitor of these proteases under the control of the *SAP1* promoter. Indeed, significantly more nodules formed on these composite roots, indicating that SAP-like proteases act as negative regulators of nodulation. More detailed and genetically refined analyses of SAP-functions are currently being performed. Furthermore, we currently conduct tissue-wide and subcellular localization studies to link these patterns to their molecular functions.

POSTER

Other Nitrogen-fixing and mycorrhizal symbioses

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INTERCELLULAR COMMUNICATION IN FREE-LIVING AND FACULTATIVE SYMBIOTIC N_2-FIXING HETEROCYSTOUS CYANOBACTERIA

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The multicellular lifestyle of filamentous, N_2-fixing heterocystous cyanobacteria requires intercellular communication, which has been studied using the model system *Anabaena* sp. PCC 7120. Proteinaceous septal junctions (gap junction analogs) are located in perforations called nanopores that traverse the septal peptidoglycan (PG) and facilitate intercellular communication[1,2]. Transmission Electron Microscopy (TEM) has shown that septal PG disks contain an array of these nanopore perforations in *Anabaena* 7120. Fluorescence Recovery After Photobleaching (FRAP) has been used to quantify intercellular communication in the cyanobacterial filaments. Another heterocystous cyanobacterium, *Richelia rhizosoleniae* (RrhiSC01), occurs in N_2-fixing symbioses with diatoms of the genus *Chaetoceros* spp. in oligotrophic oceans and produces terminal heterocysts in the filaments, as opposed to *Anabaena* 7120 that produces intercalary heterocysts[3]. In addition to FRAP and TEM of murein sacculi, we applied Fluorescence Loss In Photobleaching (FLIP) to study cell connectivity through whole filaments of *Anabaena* 7120 and RrhiSC01. A communication deficient *Anabaena* mutant (Δ*fraC*-Δ*fraD*) was used as a negative control. Heterocysts displayed slower communication compared to vegetative cells in both *Anabaena* 7120 and RrhiSC01. When normalized to cell volume, RrhiSC01 exhibited slower communication rates in both heterocysts and vegetative cells compared to *Anabaena* 7120. Additionally, the density and diameter of nanopores in both *Anabaena* 7120 and RrhiSC01 will be compared by TEM. Our findings shed light on the impact of heterocyst positioning in cell-cell communication and present a novel approach for studying intercellular communication in heterocystous cyanobacteria.

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INVESTIGATING THE ROLE OF MILDEW LOCUS O (MLO) AT THE INTERFACE OF ARBUSCULAR MYCORRHIZAL SYMBIOSIS IN *Lotus japonicus*

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Mildew Locus O (*MLO*) genes belong to a wide family involved in many plant developmental and cellular processes, such as redirection of root growth, pollen tube growth and programmed cell death, and in infection by filamentous fungi. MLO genes from clade IV, which is present in arbuscular mycorrhiza (AM)-host plants only, have been shown to modulate AM in wheat, barley and *Medicago truncatula*[1]. After decades of research about their mysterious biochemical function, MLO proteins have been recently shown to act as Ca^{2+}-permeable channels[2,3]. In this work, we focus on the role of clade IV MLO4 in AM symbiosis using *Lotus japonicus* as a model organism. We show that MLO4 promoter activity and protein localization largely overlaps with AM fungal structures in colonized *L. japonicus* hairy roots. On the other hand, MLO4 plays a broader role in *L. japonicus*, since LORE1 insertional mutants show a clear developmental root phenotype even in the absence of the AM fungus. Considering the biochemical activity of MLOs and the crucial role of Ca^{2+} signalling in the perception of diffusible symbiotic fungal signals, such as tetrameric chito-oligosaccharides (CO4) and lipo-chitooligosaccharides (LCOs), we are currently investigating the putative role of MLO4 during the chemical communication between the two symbionts. To this aim, we are monitoring intracellular Ca^{2+} changes induced by fungal molecules in *mlo4* mutant roots expressing specifically targeted aequorin-based Ca^{2+} reporters. Altogether this work provides first hints about the role of a specific *L. japonicus* MLO in the establishment and development of the AM symbiosis.

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THE ROLE OF CLATHRIN-MEDIATED ENDOCYTOSIS DURING MYC-FACTORS PERCEPTION IN ARBUSCULAR MYCORRHIZAL INTERACTION

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Clathrin-mediated endocytosis (CMA) is a major endocytic pathway in plants, driving the internalization of membrane-bound receptors. Previous studies using tyrphostin A23, a CME inhibitor, demonstrated that CME is required for the regulation of symbiotic genes in response to Nod-factors in legume-rhizobium symbiosis (Wang et al., 2015). Growing evidence indicates that this symbiosis recruited part of the signaling pathway supporting arbuscular mycorrhiza (AM). We therefore decided to investigate whether CME is also involved in AM signaling. To this aim, we treated M. truncatula roots with AM fungal signals in the absence or presence of CME inhibitors (tyrA23, Dynasore), analyzed the expression for early AM marker genes and monitored nuclear Ca2+ spiking (a hallmark of symbiotic signaling). Symbiotic gene regulation was strongly impacted by CME inhibition. Nevertheless, no significant reduction was observed in Ca2+ spiking, suggesting CME is required for gene regulation but not for upstream symbiotic signaling. We here discuss the new questions opened by such unexpected results, which contrast with our current model of symbiotic signaling in legumes.

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N_2-FIXING *VIBRIO* SP. PIGGYBACKING ON RECENTLY DISCOVERED N_2-FIXING SEAGRASS ROOT SYMBIONTS

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N_2-fixing symbioses between rhizobia and nodulating legumes are common in the terrestrial environment. Although there is some host specificity, other non-rhizobial bacteria, both N_2-fixing and non-N_2-fixing, can also be found in intact nodules[1,2]. Mohr et al.[3] recently described a terrestrial-type symbiosis between a marine gammaproteobacterium, *Ca*. Celerinatantimonas neptuna, and the seagrass *Posidonia oceanica*. The seagrass directly benefits from the nitrogen fixed in its roots, which allows the seagrass to flourish in its oligotrophic habitat, the Mediterranean Sea. Here, we used stable isotope incubations and single-cell techniques (nanoSIMS) to show that co-occurring *Vibrio* sp. also fix N_2 inside *P. oceanica* roots and may contribute to the nitrogen demand of the plant. However, amplicon sequencing of the 16S rRNA gene revealed that, in contrast to *Ca*. C. neptuna, *Vibrio* sp. were abundant in the roots only in individual plants indicating that they may be more opportunistic, similar to non-rhizobial N_2-fixing bacteria found in terrestrial root nodules[1]. Much alike *Ca*. C. neptuna, the metagenome-assembled genome (two chromosomes with 3.35 and 0.84 Mb) of the *Vibrio* sp. contained several genes/pathways commonly found in endophytes. Our combined results indicate that seagrasses are, just like terrestrial plants, capable of hosting more than one symbiotic N_2 fixer and provide further insights into the metabolic versatility and nutrient acquisition strategies in seagrass habitats.

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MICROBIOME OF THE SOYBEAN RHIZOSPHERE AND CULTIVAR-BRADYRHIZOBIUM STRAIN NODULATION COMPATIBILITY STUDY IN THE DEVELOPINT OF EFFECTIVE NITROGEN FIXING INOCULANTS

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Inoculation of soybean with exotic strains of *Bradyrhizobium japonicum* started in the mid-1960s in South Africa due to the absence of indigenous rhizobia strains that nodulate this legume. Several exotic strains were imported, screened under the local soil conditions and released as efficient commercial inoculants for soybean. However, as time went by, various complaints were raised by many farmers on inefficiency of inoculant products that fail to nodulate soybean. To investigate if the native microbial communities, among other factors[1], have a negative impact on the nodulation efficacy of *Bradyrhizobium* on soybean over the years, the microbiomes of three major soybean farms were investigated. Taxonomic hit distribution was determined using contig lowest common ancestor (contigLCA) algorism on MG-RAST[2]. The total number of species was determined as α-diversity for each microbiome from the distribution of species level annotations. The same soil from each site was used as a source of inoculum for soybeans planted in sterile sand to isolate compatible nodulating *Bradyrhizobium* strains using the soil trap experiment. The trapped *Bradyrhizobium* strains were re-inoculated on various soybean cultivars for a nodulation compatibility and nitrogen fixation efficiency evaluations. The study generated various data with considerable importance in the quest for selection and development of effective *Bradyrhizobium* inoculants for use on different soybean cultivars in South Africa.

Key words: Nitrogen-fixation, Nodulation, soybean, Bradyrhizobium

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SIGNALLING BETWEEN PLANTS AND ENDOPHYTIC DIAZOTROPHIC BACTERIA: A VIEW FROM THE PLANT SIDE

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One of the great challenges of this century is to increase agricultural production in a sustainable way. However, the intensification of extreme climate change events is affecting agriculture and reducing crop productivity. The associations that occur between economically important crops, especially monocots, with associative and endophytic diazotrophic bacteria have raised a large interest in their use in agriculture, in view of the positive effects on the promotion of plant growth, as well as increase in tolerance against biotic and abiotic stresses. Bioinoculants of associative and endophytic diazotrophic bacteria had been shown to lead to positive results on crop 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 expression profiles involved in metabolism, growth and development controlled by nitrogen, water and endophytic nitrogen-fixing bacteria during a successful association. Functional analyses of plant genes are being performed.

Altogether, 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. A second level of regulation might involve cell division and cell wall synthesis controls, hormonal regulation and reprograming of nitrogen metabolism. Several of the genetic controls and expression profiles might possibly be used as tools for optimization of plant growth and response to bioinoculants, presenting a sustainable alternative to the use of chemical fertilizers, with positive economic and environmental impacts on agriculture.

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TISSUE-SPECIFIC TRAP-SEQ FROM ARBUSCULAR MYCORRHIZAL *L. JAPONICUS* ROOTS

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RNA translation is crucial in gene expression during development or adaptation to environmental changes in eukaryote organisms[1]. Plant roots are able to adapt to the rhizosphere environment to maximize nutrient and water acquisition. The mutualistic association between plant and arbuscular mycorrhiza (AM) fungi enhances nutrition for both symbionts; the plant benefits from mineral nutrients, mainly phosphate, and the fungi gain photosynthetically-fixed carbon in the form of lipids and sugars[2,3,4]. AM development involves cell-autonomous transcriptional reprogramming and subcellular remolding. However, this process is not synchronized; all stages of AM development are simultaneously present in the root[5]. Therefore, the development of methods to study cell-type specific mRNA profiles in AM roots is needed. Here we established tissue-specific Translating Ribosome Affinity Purification (TRAP) for *Lotus japonicus* roots colonized by the AM fungus *Rhizophagus irregularis* and combined it with RNA-seq to study the tissue-specific translatomes during AM. We identified hundreds of differentially enriched epidermis- or cortex-specific transcripts in ribosomes, including mRNAs of genes involved in AM formation. In addition, we identified 62 genes associated specifically with the ribosome in the presence of the AM fungi. Some of these mRNAs encode proteins involved in transmembrane transport, lipid metabolism and transcription regulation. These changes in the translatome likely contribute to the reprogramming of root cells for AM symbiosis.

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GROWTH RESPONSE AND BACTERIAL COLONIZATION OF TWO *UROCHLOA* CULTIVARS INOCULATED WITH *AZOSPIRILLUM BALDANIORUM*

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Urochloa species are the most planted forage used for cattle production in the tropics. Plant-bacteria interaction is cultivar dependent, and a better understanding of this fine-tuning can be useful to improve the plant growth benefits of this association. The current study aimed at testing the performance of two cultivars, *U. decumbens* (cv. Basilisk) and *U. humidicola* (cv. Llanero), inoculated with *Azospirillum baldaniorum* Ab-Sp245^T by evaluating plant initial growth and root colonization. Experiments using Hoagland's solution, with and without N as nitrate (0-, 0.3-, and 3-mM N) to measure plant growth response after two cuts in 66 days and without N for bacterial colonization using confocal laser scanning microscopy technique with GFP-labelled bacterium. *Urocloa* response to Ab-Sp245 inoculation was modified by the cultivar tested and N level. The 0.3 mm N associated with Ab-Sp245^T improves plant growth response and N accumulation. Bacterial colonization also differs between the cultivars tested being cv. Basilisk, the responsive one, colonized by the target bacterium in all root parts but only superficially and in cv. Llanero was observed colonizing only the root hairs and in low bacterial density. Growth response and bacterial colonization were positively correlated, and benefits can be visualized by higher biomass production and N accumulated in a shorter period of growth.

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HEAT AND CHEMICAL THERAPY ARE USED TO REDUCE THE NATURAL DIAZOTROPHIC POPULATION IN SUGARCANE STEM CUTTING AS AN OPPORTUNITY TO INTRODUCE NEWLY SELECTED STRAINS

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The inoculation of diazotrophs in sugarcane is difficult as the plant is vegetatively propagated, so quantifying the contribution of a new bacterial community is impaired by the natural inhabitants. To overcome this difficulty, the heat treatment (HT) used to control the ratoon stunt disease was modified using a chemical step with acetic acid (AC- 2%) as a biocide. Four experiments were performed using two sugarcane cultivars, inoculated or not with five diazotrophs. The treatments tested were the control, HT (52ºC for 30'); HT plus Ac (2% at 52ºC for 10', and 10'HT + 10'Ac room temperature), and inoculation using the five bacterial strains. Plant and bacterial growth (two methods) and nitrogenase activity were used. The reduction of 20 min of the HT and the addition of Ac controls the natural diazotrophic community and establishes the five diazotrophs, maintaining growth and increasing 3.4 times ARA activity over the traditional HT.

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UNCOVERING PLANT MICROBIOMES USING LONG-READ METAGENOMIC SEQUENCING

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The microbiome of plants plays a pivotal role in their growth and health. Despite its importance, many fundamental questions about the microbiome remain largely unanswered, such as the identification of colonizing bacterial species, the genes they carry, and the location of these genes on chromosomes or plasmids. To gain insights into the genetic makeup of the rice leaf microbiome, we performed a metagenomic analysis using long-read sequences, and developed a genomic DNA extraction method that provides relatively intact DNA for long-read sequencing. 1.8 Gb reads were assembled into 26,067 contigs, including 136 circular sequences of less than 1 Mbp, as well as 172 large (≥ 1 Mbp) sequences, six of which were circularized. Within these contigs, 669 complete 16S rRNA genes were clustered into 166 bacterial species, 130 of which showed low identity to previously defined sequences, suggesting that they represent novel species. The large circular contigs contain novel chromosomes and a megaplasmid, and most of the smaller circular contigs (<1 Mbp) were defined as novel plasmids or bacteriophages. One circular contig represents the complete chromosome of an uncultivated bacterium in the candidate phylum Candidatus Saccharibacteria. Our findings demonstrate the efficacy of long-read-based metagenomics for profiling microbial communities and discovering novel sequences in plant-microbiome studies.

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MARINE N_2-FIXING BACTERIUM IN SEAGRASS ROOTS ECHOES TERRESTRIAL PLANT SYMBIOSES

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Nitrogen (N_2)-fixing microorganisms are crucial to the supply of nitrogen in a variety of ecosystems[1,2,3]. In the terrestrial environment, N_2-fixing symbioses between bacteria and plants are widespread and enable plants to grow in nitrogen-poor soils. Many of these symbioses are quite intimate and rely on a mutual exchange of nitrogen and carbon compounds[2]. We recently discovered an N_2-fixing root endophyte in *Posidonia oceanica*[4], a contemporary seagrass whose ancestors evolved from terrestrial flowering plants about 100 million years ago. Similar to its terrestrial counterparts, the N_2-fixing bacterium, named *Candidatus* Celerinatantimonas neptuna, supplies ammonium and amino acids to its seagrass host, which supplies sugars in return. Genomic evidence indicates that the bacterial symbiont may also promote plant growth via the production of hormones, siderophores or antimicrobial compounds, traits commonly found in terrestrial endophytes[5]. Yet, the novel symbiont is of marine origin and as such may have aided the migration of flowering plants into the sea. Relatives of *Ca*. C. neptuna are present in coastal ecosystems worldwide, where they may form similar symbioses with other seagrasses or salt marsh plants, allowing them to thrive in nutrient poor ecosystems.

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CARBON UPTAKE MECHANISMS SUPPORTING THE N_2-FIXING DIATOM ENDOSYMBIONTS *Richelia* SPP.

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Endosymbiotic N_2-fixing, heterocyst-forming cyanobacteria of the genus *Richelia* provide some diatoms with fixed nitrogen in oligotrophic areas of the oceans[1]. *R. euintracellularis* (ReuHH01) is a cytoplasmic endosymbiont of *Hemiaulus hauckii*, whereas *R. intracellularis* (RintRC01) is a periplasmic endosymbiont of *Rhizosolenia clevei*. Because N_2 fixation is dependent on reduced carbon compounds and both *Richelia* species appear capable of CO_2 fixation[2], we looked for organic carbon and bicarbonate transporter genes in the genomes of the endosymbionts. Both ReuHH01 and RintRC01 bear genes for an ABC transporter for glucosides and invertases. We showed that the ReuHH01 ABC substrate binding protein binds sucrose with high affinity and that the ReuHH01 invertase is a neutral invertase that splits sucrose to fructose and glucose. Both ReuHH01 and RintRC01 also bear genes for SulP family transporters, which are involved in the uptake of anions such as sulfate, bicarbonate, nitrate or molybdate. To test their possible activity for bicarbonate uptake, we inserted those genes (one from ReuHH01; three from RintRC01) in a mutant of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 that lacks any bicarbonate or CO_2 uptake system and requires high concentrations of inorganic C for growth (mutant strain D5). Only RintRC_3892 (BicA) provided strain D5 with the ability to grow under air levels of CO_2. Strain D5 expressing RintRC_3892 showed Na^+-dependent ^{14}C-bicarbonate uptake with a $K_{0.5}$ value for bicarbonate of about 1 mM. The expression of relevant genes in wild populations from the North Atlantic and South China Sea was also tested and could be corroborated. Our results illustrate the utility of heterologous gene expression to identify endosymbiont functions and highlights aspects of C transport and metabolism important to support N_2 fixation in the *Richelia* endosymbionts.

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THE ROLE OF THE *MYB17* GENE IN THE LIGNIFICATION OF CELL WALLS IN INFECTED NODULES OF *CASUARINA GLAUCA*

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The actinobacterium *Frankia* can form symbiotic relationships with a diverse group of plants known collectively as actinorhizal plants, leading to the formation of nitrogen-fixing root nodules. While nitrogen fixation by nitrogenase requires energy best attained via aerobic respiration, the enzyme is also irreversibly denatured by O_2, resulting in an oxygen dilemma. In legumes, the plant protects nitrogenase from O_2; *Frankia*, on the other hand, provides its own O_2 protection in most actinorhizal nodules – with the exception of nodules induced on *Casuarina* spp. Here, infected cells are surrounded by lignin in their primary cell walls, which prevents O_2 from reaching the cytosol and thus, *Frankia*. Several MYB transcription factors are known to upregulate monolignol biosynthesis genes, and a specific *MYB* gene, *CgMYB17*, was observed to be upregulated in *C. glauca* nodules, while its ortholog in the actinorhizal relative *Alnus glutinosa*, where the walls of infected cells do not contain lignin, was not upregulated in nodules[1]. Therefore, we tried to verify whether *CgMYB17* was responsible for the lignification of infected nodule cells in *C. glauca*. Expression levels of *CgMYB17* in different organs of *C. glauca* were compared to those of genes encoding enzymes involved in lignin biosynthesis via RT-qPCR. *CgMYB17* was expressed under the *35S* promoter in *Nicotiana benthamiana* leaves by agroinfiltration, followed by lignin staining, showing increased lignification of minor veins. A transcriptional fusion of the *CgMYB17* promoter with a GFP reporter was introduced in the legume *Lotus japonicus* by hairy root transformation to analyse organ- and cell type specific expression was transferrable to legumes.

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PHOTOPHYSIOLOGY OF SYMBIOSIS BETWEEN HAPTOPHYTE HOST AND UCYN-A DIAZOTROPH

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The diazotrophic cyanobacterium UCYN-A shows unusual degree of metabolic streamlining[1] suggesting obligate symbiosis with its haptophyte host *Braarudosphaera bigelowii*[2]. Here we report results of the laboratory experiment studying the metabolic coupling between the host and UCYN-A. Laboratory cultures[3] were grown at 18°C under 12h Light/ 12h Dark cycles. Photosynthesis of the host was assayed by Chlorophyll variable fluorescence (FRR fluorometery) and by oxygen production (Clark electrode). Nitrogen fixation of UCYN-A was measured as acetylene reduction by GC.

N_2 fixation in UCYN-A occurs only during the light period and is strictly light dependent and stops immediately when cells are transferred into dark. The light-dependent rate of N_2 fixation in UCYN-A saturates at light intensities of 50-70 umol quanta.m^{-2}.s^{-1}, independently of the growth irradiance. This is significantly lower than the saturation of the photosynthesis of the host (120-250 umol quanta.m^{-2}.s^{-1}, depending on the growth irradiance). N_2 fixation of the symbiont seems to be under circadian rhythm, cells do not fix N_2 when exposed to light during the subjective night period. The light-dependent N_2 fixation of the symbiont continues for several hours even if the photosynthesis of the host is fully inhibited by DCMU. This indicates that there exists a pool of reduced carbon produced by the host that fuels N_2 fixation even when its photosynthesis is inhibited. On the other hand, N_2 fixation can be inhibited by compounds that block thylakoid membrane electron flow in the symbiont or that collapse the transmembrane gradient. Our results confirm the active and indispensable role of Photosystem 1 in supplying reductant and/or ATP to N_2 fixation in UCYN-A.

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MYCORRHIZA IMPROVES SOYBEAN GROWTH EVEN IN NUTRIENT-RICH SOILS OF CENTRAL EUROPE, BUT INDEPENDENTLY OF *BRADYRHIZOBIUM* INOCULATION

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Symbiotic microorganisms, rhizobia and arbuscular mycorrhizal fungi (AMF) improve plant growth and may be of use for maintaining and even restoring soil. However, data on the latter function are sparse and only indirect. Our study focused upon potential application of a widespread AMF species, *Funneliformis mosseae*, in production of *Glycine max*. Working in greenhouse conditions, we examined whether in a nutrient-sufficient environment of Central Europe AMF would improve crop biomass accumulation and nutrition, as well as stability of soil aggregates (SAS). We also looked for synergistic effect of dual inoculation using both AMF and symbiotic rhizobium. Plants were or were not inoculated with AMF or *Bradyrhizobium japonicum* in a two-factorial design. AMF inoculation increased soybean biomass and N content, but P content in shoots was not affected. Mycorrhiza did not affect either glomalins abundance or SAS. All the impacts were independent of rhizobial inoculation, although it decreased the AMF abundance in soybean roots. Our assay suggests that arbuscular mycorrhiza may have positive effect on soybean growth under high-loaded management. Positive effects of AMF on soybean growth, together with the fact that AMF generally do not thrive in high nutrient availability, should be taken into account when planning mineral fertilization levels.

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CRISPR-ASSOCIATED TRANSPOSONS (CAST) TO GENOME EDIT *Nostoc azollae* AND OTHER N_2-FIXING CYANOBACTERIA

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Floating ferns from the genus *Azolla* are in a symbiotic association with the N_2-fixing, heterocyst-forming filamentous cyanobacterium *Nostoc azollae* which provides the ferns with fixed nitrogen, allowing them to prosper without nitrogen fertilizers. *N. azollae* is an obligatory symbiont with an eroded genome whose life cycle is tightly coordinated with that of its host [1]. Consequently, its culture under laboratory conditions has proved impossible preventing stable genetic editing. Here, we develop tools for efficient RNA-guided transposition with the CAST system [2] in the characteristically polyploid cells of N_2-fixing cyanobacteria: the filamentous heterocyst-forming *Anabaena* sp. PCC 7120 and *Nostoc punctiforme* PCC73102, and the unicellular marine cyanobacterium *Crocosphaera subtropica* 41152, with the ultimate goal of editing and tagging genes of *N. azollae in planta*. In order to enhance sharing and modular cloning, the CAST-encoding genes, sgRNA, Tn7 borders, and specific cyanobacterial promoters used were customised into GoldenGate level 0 modules [3]. The single-guide (sg) RNAs that bind the Cas12k were designed to target the widely used *gfp* in *Anabaena* mutant strains CSVT15 (*amt1*::*gfp*), CSAM137 (*sepJ*::*gfp*), *psbA2* and the well conserved *nifK* gene in the cyanobacteria. The experiments showed that the system, provided the sgRNA encoded leading strand of the targeted gene, is highly efficient in creating genetic modifications with fluorescent tags or selection genes. This new tool has the potential to greatly advance genome editing in *N. azollae* and other N_2-fixing cyanobacteria, as well as setting the groundwork for streamlined and precise genetic manipulation of cyanobacteria involved in other symbiotic relationships and even more complex ecological communities.

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DEVELOPMENT OF BIODEGRADABLE COATING FOR SOYBEAN SEEDS AND THEIR APPLICATION FOR BRADYRHIZOBIUM JAPONICUM IMMOBILIZATION

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Commercial interest in biological alternatives to chemical applications in agriculture remains high and is growing [1]. Barriers to the use of these technologies remain, including cost, survival of the bacterial inoculant, and mode of application. Seed inoculation with BNF is an ideal tool for delivering high densities of viable beneficial microorganisms to the soil, where they can be colonized by emerging plant roots. However, despite numerous studies demonstrating the ability of beneficial microorganisms to enhance plant development, few seed-attached bioinoculants are commercially available because maintaining viable microorganisms during seed treatment and storage is a major concern [2]. In this scenario, polyglycerol citrate (PGCit), a biodegradable aliphatic polyester produced by the polycondensation of citric acid and glycerol, is emerging as a new class of polymer for this application. In this work, the synthesis and application of PGCit as a new liquid inoculant for soybean seed coating were investigated. Seed coating with PGCit improved Bradyrhizobium survival on seeds stored for more than 14 days, contributing to successful bacterial colonization and plant development. Therefore, the seed coating technique used in the present work could be considered a promising environmentally friendly approach to improve soybean production through the use of a microbial inoculant. Greenhouse studies have confirmed its beneficial effect on soybean, while the polymer coating protects bacteria and seeds from abiotic environmental stress and promotes successful inoculant colonization, as measured by the number of nodules counted after harvesting the soybean plants.

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BURKHOLDERIA VIETNAMIENSIS STRAIN AAR-N445 AS A POTENTIAL NITROGEN-FIXING ENDOPHYTE FOR *ELAEIS GUINEENSIS* THROUGH CARBON SOURCE OPTIMISATION

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The provision of carbon supply by the host plant plays a crucial role in improving bacterial nitrogen fixation. However, which carbon source is most effective in oil palm remains unclear. Endophyte derives support for growth from non-structural carbohydrates, amino acids, and inorganic nutrients present in the intercellular spaces, and an increase in sugar contents in plants leads to a significant boost in endophytic nitrogen-fixing activity[1]. In legumes, dicarboxylates offer an essential carbon source to Bacteroides, which further provides fixed nitrogen to the host plant[2]. It is, therefore, imperative to investigate the impact of carbon sources on bacterial nitrogen fixation in non-legume crops such as oil palm. This study aims to modify N-free media with different non-structural carbohydrate components resembling metabolite profiles of oil palm tissues that would favour the identification of oil palm-competent N-fixing bacteria.

Amino acid profiling in older palms showed higher amino acid levels than in younger palms, while total sugars did not differ significantly. Sucrose content was highest regardless of palm age. The modification of sugar content in N-free media, resembling oil palm tissues, led to the identification of isolate AAR-N445 being the best strain, which produced the highest nitrogenase activity. The nitrogenase activity was on par with the preferable substrates' utilization of AAR-N445 from GEN III Microplates™. *nifH* targeted amplicon analysis of 6-month-old palms with AAR-N445 inoculation showed an increase of *nifH* gene abundances with the highest in the leaf samples. This study provides new insights into the importance of carbon sources for nitrogen fixation activity in oil palm, which may have significant implications for sustainable production.

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BACILLUS VELEZENSIS S141: INSIGHTS DUAL ACTIONS: PLANT GROWTH-PROMOTION AND BIOCONTROL AGENT IN LEGUMES

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In *Bacillus velezensis* strain S141 has been identified as a plant growth-promoting rhizobacterium for soybean, enhancing growth, nodulation, and nitrogen fixation efficiency by coinoculation with *Bradyrhizobium diazoefficiens* USDA110. The disruption of genes related to IAA and cytokinin biosynthesis reduced the number of large and very large size nodules. S141 also exhibits strong antifungal activity against *Cercospora canescens* PAK1 (causing agent of leaf spot disease in munbean) through multiple enzymatic hydrolases and secondary metabolites from secreted compounds. Testing the cell-free secretions from a single culture of S141 and dual culture of S141+PAK1 showed inhibition of fungal growth. The biocontrol efficiency of S141 against *Cercospora* leaf spot on mungbean was also evaluated, with a control efficiency of 83% after 2 days of infection. The study identified differentially expressed genes involved in biosynthetic genes encoding enzymatic hydrolases, such as protease, β-glucanase, and N-acyl glucosaminase, and genes related to secondary metabolites. These findings suggest that S141 can be further developed as biocontrol agent against *C. canescens* and superior co-inoculants with rhizobium for economic legumes production in order to apply less chemical-N fungicide.

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CHEMICAL COMMUNICATION DURING FEATHERMOSS-CYANOBACTERIA SYMBIOSIS IN BOREAL FORESTS

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Atmospheric di-nitrogen fixing cyanobacteria are primary nitrogen input sources in nitrogen-limited boreal forests[1,2]. The non-vascular feathermosses (*Pleurozium schreberi*, *Hylocomium splendens* and *Ptilium crista-castrensis*) host epiphytical cyanobacterial communities on their leaf surface[1,3]. The mosses benefit from this association by acquiring the fixed-nitrogen from the symbiont[1]. This highly beneficial interaction is the outcome of a cascade of signal exchanges between the engaging partners[4]. However, the molecular mechanisms underlying these signaling events, their temporal occurrence and the chemicals (e.g. metabolites) which mediate this symbiosis are yet to be unraveled in detail. We developed an experimental study system, through which we over time are characterizing different stages in the chemical and physical interaction between these feathermoss species and a symbiotically competent cyanobacteria strain (*Nostoc* sp.), under axenic conditions. Untargeted metabolomic analysis using LC-MS/MS is used to identify potential metabolite signals, which facilitate hormogonia induction, chemotaxis and di-nitrogen fixation by cyanobacteria. The ultimate goal of this research is to provide biomarkers using this model system, for nitrogen enrichment in boreal forests and that could potentially be translated to benefit other ecosystems.

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BEYOND *NIF* GENES: DIAZOTROPH GENE EXPRESSION *IN PLANTA* DURING NITROGEN FIXATION IN MAIZE

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Extending symbiotic N-fixation to cereal crops is considered the holy grail of agricultural production[1]. It has been suggested that maize aerial root mucilage may be a model to determine the host-microbe interactions that impact diazotrophic symbioses in cereals[2]. Recently, Sierra Mixe maize aerial root mucilage was determined to harbor a diazotrophic microbiome that provides 28-82% of the plant's nitrogen[3]. Genomic characterization of Sierra Mixe maize N-fixing microbial isolates found that some of the best N-fixers did not have the minimal *nif* gene set (*nifHDKENB*) thought to be required for diazotrophy[4, 5]. Non-canonical genes for N-fixation are currently unknown but seem to exist in a variety of organisms. In this study, we used metatranscriptomics with RNA-Seq from field-grown Sierra Mixe maize aerial root mucilage, separated microbial reads, assembled and annotated transcripts, and analyzed the differential gene expression relative to the amount of fixed nitrogen in the source plants. We found that a small subset of the minimal *nif* gene group was expressed in aerial root mucilage from plants in two conditions (high and low nitrogen). However, other *nif* genes (*nifS, nifU, nifV*) and their regulators (*nifA, nifL*) as well as alternative nitrogenase genes were expressed, suggesting that additional genes are needed for N-fixation in this system as compared to the minimal gene set. Additionally, glycan-digestive genes were expressed, indicating that mucilage sugars are available to fuel N-fixation. Our findings suggest the presence of alternative mechanisms for N-fixation in aerial root mucilage, beyond the canonical *nif* genes. This study broadens our understanding of N-fixation in cereal-associated microbes and opens new avenues for research on sustainable agricultural practices.

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INVESTIGATING COPPER TOLERANCE IN *FRANKIA INEFFICAX* EUI1C THROUGH TARGETED MUTAGENESIS

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Heavy metal contamination in soils is a major environmental concern that negatively impacts ecosystem biodiversity and productivity. Phytoremediation is a promising strategy for the sustainable restoration of degraded soils, and actinorhizal plants are particularly useful as pioneer species. When associated with *Frankia*, the adverse effects of heavy metal pollution are mitigated, which aids in phytoremediation performance and survival of actinorhizal plants. Previous studies have shown that the strain *Frankia inefficax* EuI1c can tolerate up to 5.0 mM of copper, and its resistance mechanisms are hypothesized to mimic those of heavy metal extremophiles. Inoculation studies were performed previously using alder seedlings treated with a 1.0 mM copper solution. After 10 weeks of growth, seedling survival was significantly increased in seedlings inoculated with *F. inefficax* compared to seedlings inoculated with an alder-associating, non-copper tolerant *Frankia* strain ($p = 0.02$). However, the genetic tools responsible for this observed tolerance have yet to be characterized.

To gain a better understanding of the genetic mechanisms underlying copper tolerance in *F. inefficax,* several genes that are hypothesized to contribute to this tolerance have been targeted for deletion. Generation of *Frankia* mutants has historically been limited to non-targeted or transient transformants. Recently, a stable transformation method through conjugation was developed, allowing for CRISPR-Cas9 gene editing in *Frankia*. Using this method, plasmid constructs have been generated to target three genes in *F. inefficax*: FraEuI1c_1869, a putative *copD* gene, FraEuI1c_6307, a putative *copA* gene, and FraEuI1c_1092, which likely encodes for a p-type ATPase. Generation of these mutant *F. inefficax* strains will allow for the downstream characterization of the mechanisms used by this strain to survive, and subsequently aid the health and survival of actinorhizal hosts, in soils degraded by excess concentrations of heavy metals.

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A SIMPLE AND EFFICIENT PROTOCOL FOR GENERATING TRANSGENIC HAIRY ROOTS USING *AGROBACTERIUM RHIZOGENES*

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For decades, *Agrobacterium rhizogenes* (now *Rhizobium rhizogenes*), the causative agent of hairy root disease, has been harnessed as an interkingdom DNA-delivery tool for generating transgenic hairy roots on a wide variety of plants. One of the strategies involves the construction of transconjugant *R. rhizogenes* by transferring gene(s) of interest into previously constructed *R. rhizogenes* pBR322 acceptor strains; little has been done, however, to improve upon this system since its implementation.

We developed a simplified method utilising bi-parental mating in conjunction with effective counterselection for generating *R. rhizogenes* transconjugants. Central to this was the construction of a golden gate-compatible pBR322-derived integration vector (pIV101). Although this protocol remains limited to pBR322-acceptor strains, pIV101 facilitated an efficient construction of recombinant vectors, effective screening of transconjugants, and RP4-based mobilisation compatibility that enabled simplified conjugal transfer. Transconjugants from this system were tested on *Lotus japonicus* and found to be efficient for the transformation of transgenic hairy roots and supported infection of nodules by a rhizobia symbiont. With this new pIV101 vector, one decreased both the time and labour needed to create transconjugant *R. rhizogenes* for the subsequent transgenic hairy root transformation of *Lotus japonicus,* and it could readily be applied to the transformation of other plants.

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RAPID GENETIC SCREENING OF BARLEY ENGINEERED LINES SUGGEST A PUTATIVE AUXIN RESPONSIVE MEDICAGO PROMOTER OPERATING IN BARLEY

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Most plants cannot fix atmospheric nitrogen directly from the air and must rely on natural processes that convert atmospheric nitrogen into bioavailable nitrogen pools. In contrast, synthetic processes have become the primary nitrogen source for agriculture and play a central role in worldwide food security. Plants such as legumes have evolved symbiotic relationships with nitrogen-fixing bacteria that provide nitrogen to the plants in exchange for carbon supply. This symbiotic exchange occurs in root nodules capable of harbouring the symbionts and creating the conditions for bacterial nitrogen fixation. We aim to engineer nitrogen-fixing root nodulation into barley, and to transfer from there to other cereal crops. For that purpose and to better understand how legume nodulation related genes interact with barley native genes we have used both rapid and standard stable transformation methods for screening of engineered lines and to conduct molecular analyses of the Medicago *NODULE INCEPTION (NIN)* gene. *NIN* is a central regulator of nitrogen-fixing root nodulation conserved across various nodulating species shown to be involved in multiple stages of root nodule organogenesis.

Maize optimized *MtNIN* was overexpressed in barley concomitantly with two multiplexed transactivation assay readouts *pMtCRE1::FLAG::GUS* and *pMtLb1::6xHIS::GUS* using the STAble Root Transformation System (STARTS) and also commonly used standard stable transformation method. Both promoter readouts were chosen as they are involved in early (the former; promoter of *CYTOKININ RESPONSE 1*) and late (the latter; promoter of *LEGHEMOGLOBIN 1*) nodulation stages. Preliminary GUS staining results from stable transformants grown in soil conclusively indicate that expression of *MtNIN* driven by *pZmUBI*, *pAtUBI*, and *pLjUBI* does not induce the expression of *pMtCRE1* and *pMtLb1* in barley. This implies that additional factors are required for NIN to activate target promoters and that *NIN* alone is not sufficient.

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NOVEL INSIGHTS INTO THE REGULATION OF NITROGEN FIXATION

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The large energetic requirements of nitrogen fixation foresee sophisticated regulatory networks to allow diazotrophic organisms to thrive in competitive environments. In some free-living diazotrophic Proteobacteria, the expression of the *nif* genes, encoding the Mo-dependant nitrogenase, is controlled by a non-canonical two-component system, the NifL-NifA system [1]. Unlike other two-component systems, the regulatory outputs depend on stoichiometric interactions between NifL, an anti-activator protein, and NifA, the master transcriptional activator of *nif* genes. NifL can perceive the signals of excess oxygen and low energy via the binding of FAD to the PAS1 domain and ADP to the GHKL domain in its N- and C-termini, respectively. NifA also responds to environmental stimuli, as its regulatory GAF domain binds 2-oxoglutarate (a metabolite in the interface of carbon and nitrogen metabolism), enabling the escape from NifL inhibition. GlnK, a nitrogen status sensor, can also interact with this system to form a ternary complex GlnK-NifL-NifA when nitrogen is in excess, and GlnK is non-uridylylated. In my talk, I will share how the mechanistic understanding of the NifL-NifA regulation led to the development of a strategy to generate strains able to secrete ammonia [2]. In addition, I will share recent developments on the purification of these protein complexes and the use of cryoEM, 3D modelling and extensive mutagenesis for their structural characterisation. Our preliminary structural analysis of the NifL-NifA complex uncovers exciting mechanistic cues. Overall, these developments are guiding engineering efforts to generate nitrogen-fixing organisms with an increased ability to release ammonia for the benefit of plant crops.

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ASSESSMENT OF NITROGEN FIXATION AND GENETIC MALLEABILITY OF TWO BARLEY ASSOCIATED DIAZOTROPHS

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With fertiliser application being unsustainable and highly polluting, alternative approaches to supplement crop plant demand for nitrogen are needed. Biological Nitrogen Fixation (BNF), performed by free-living diazotrophic bacteria in soils, offers an alternative source of nitrogen for crops. Following a screen that grew barley (*Hordeum vulgare* cv. Golden Promise) in unsterilised soil sourced from Wytham Wood (Oxfordshire), two bacteria (B6 and J2) from the genus *Ciceribacter* were found to form associations with barley roots and could potentially fix nitrogen. Whole genome sequencing of these strains showed they possess classical nitrogen-fixing genes found in other free-living fixers. Using acetylene reduction assays to quantify rates of nitrogen fixation, the two strains' ability to fix in both free-living and *in-planta* conditions was shown to be significantly higher than other model diazotrophs under similar experimental conditions[1]. Strains B6 and J2 were also conjugated with rhizopine receiver plasmids pSIR02 and pSIR05[2] (*scyllo*-inosamine – natural chemical signals synthesised in legume nodules) and were able to perceive exogenous rhizopine at 10 µM.

These findings are highly significant as they show these bacteria can be synthetically engineered to perceive plant root signals and respond to them, enabling a targeted system of interaction between specific plant hosts and bacteria. With these strains also being able to fix high levels of nitrogen relative to other know fixers, these bacteria are prime candidates for further engineering with traits suited to agricultural systems to facilitate sustainable crop production.

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ASSEMBLY OF NITROGENASE BIOSYNTHETIC PATHWAY IN *SACCHAROMYCES CEREVISIAE* BY USING 2A PEPTIDES

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Nitrogenase in some bacteria and archaea catalyzes conversion of N_2 to ammonia. To reconstitute a nitrogenase biosynthetic pathway in a eukaryotic host is still a challenge, since synthesis of nitrogenase requires a large number of *nif* (*ni*trogen *f*ixation) genes. 2A peptides, the ‘‘self-cleaving’’ small (18–22 amino acids) peptides, are used to express multiple proteins from a single open reading frame (ORF) in eukaryotic cells [1] (Ryan et al., 1999).

To test whether 2A peptide was able of separating bacterial nitrogenase proteins in ***Saccharomyces cerevisiae***, two Nif proteins (NifB and NifH), which are the first and second proteins in Nif-cluster (NifBHDKENXHesANifV) of *Paenibacillus polymyxa* WLY78 [2], were chosen to be tested. Western blot showed that the cleavage efficiency of NifB-2A-NifH polyprotein linked by four different 2A peptides (P2A, T2A, E2A and F2A) in *S. cerevisiae* ranges from ~50% to ~90% [3]. The presence of a 2A tail in NifB, NifH and NifD did not affect their activity, but the presence of a 2A tail decreased NifK activity. Then, nine Nif proteins (NifB, NifH, NifD, NifK, NifE, NifN, NifX, HesA and NifV) from *P. polymyxa* WLY78 are fused into two huge proteins and then they are co-expressed in *S. cerevisiae*. Western blot revealed that each of the nine Nif proteins was expressed in *S. cerevisiae.* Furthermore, *P. polymyxa* NifH and *Klebsiella oxytoca* NifS and NifU are fused into a polyprotein and co-expressed in *S. cerevisiae*, and the expressed NifH from the huge protein (NifS-2A-NifU-2A-NifH) exhibits Fe activity [3]. This study shows the potential of utilization of 2A peptides to express multicomponent nitrogenase system in *S. cerevisiae* and other eukaryotic cells.

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DESIGNING LIGHT-UTILISING NITROGENASE (LUN) BY COMPARATIVE AND STRUCTURAL BASIS OF LIGHT-DRIVEN PROTOCHLOROPHYLLIDE OXIDOREDUCTASE (LPOR)

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The three-dimensional structure of light-dependent protochlorophyllide oxidoreductase (LPOR) has recently been solved[1]. Using this model, we have previously identified the substrate binding mode in the Pchlide-NADPH-LPOR complex, and explored the reaction mechanism using quantum-chemical methods[2]. These studies revealed how the LPOR active site can promote the photo-driven reduction through the initial electron-transfer from Tyr189 to the substrate, followed by hydride transfer from NADPH and internal electron-transfer from Cys222 to the deprotonated Tyr189 radical[2].

Since the key electron-transfer event that initiates catalysis in LPOR is made possible, in a large part, by the active-side amino acids themselves (rather than only the interplay of NADPH and PChlide molecular orbitals), we hypothesized that those active site features might be sufficient to enable light-induced electron transfer to other chemical entities than Pchlide. Combined with the knowledge that nitrogenase and the dark-operative Pchlide-oxydoreductase enzyme (DPOR) are structurally related, and that in the previously solved DPOR complex structure the substrate Pchlide is found to be almost exactly in the position where FeMoco in nitrogenase complex would be located, we hypothesized that the LPOR scaffold might be able to accommodate FeMoco and catalyze the light-driven electron-transfer from Tyr189 to the FeMoco. We therefore computationally replaced Pchlide in LPOR with FeMoco to test whether this artificial LPOR-FeMoco would be stable enough to enable its possible use as a light-driven nitrogen fixation system, in a quest for a possible light-utilizing nitrogenase (LUN)[3].

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ENGINEERING FUNCTIONAL NITROGENASE COFACTOR BIOSYNTHESIS PROTEIN NIFEN IN YEAST

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The biosynthesis of the iron-molybdenum cofactor (FeMo-co), a [7Fe-9S-C-Mo-*R*-homocitrate] cluster located at the active site of Mo-type nitrogenase, requires the productive interactions of several nitrogen fixation (*nif*) gene products.[1] At the center of the FeMo-co biosynthetic pathway is the scaffolding protein NifEN. NifEN accepts an [8Fe-9S-C] cluster produced by NifB, designated NifB-co, and incorporates the organic moiety homocitrate (product of NifV) and a Mo atom supplied by NifQ to generate FeMo-co in a series of reactions that additionally require NifH. The structural complexity of FeMo-co and its susceptibility of towards oxygen, together with the number of genetic determinants involved in its biosynthesis, make its production in recombinant eukaryotic cells difficult. This, in turn, poses an obstacle to the engineering crops capable of harvesting atmospheric N_2 for use as nitrogen source.[2]

In this work we have identified two NifEN variants that formed soluble and functional complexes when targeted to mitochondria in aerobically cultured *Saccharomyces cerevisiae*. The isolated protein produced FeMo-co *in vitro* when combined with NifB and NifH also produced in yeast and manifests ongoing progress in the generation of nitrogen-fixing plants.

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LIGAND BINDING SPECIFICITIES AND RECEPTOR COMPLEX FORMATION IN LEGUME FRIEND-OR-FOE RECOGNITION

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LysM receptor kinases (LysM-RK) are central to both symbiotic and pathogenic interactions between plants and microorganisms. They are key players in chitin-based 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. Understanding ligand binding specificity and selectivity is important for future approaches to engineer root nodule symbiosis.
In an extension of our previous studies [1,2,3], we are further investigating and dissecting the different ligand binding sites, affinities and specificities of LysM-RKs involved in symbiosis, chitin-elicited defense, or both, by biochemistry and structural biology techniques. Insights into this core receptor complexes and how ligand binding initiates formation of functional signaling complexes by ligand-induced dimerization supports the ongoing endeavours in engineering symbiotic nitrogen fixation into cereals and also increases understanding of pathogen defense pathways.

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IMPROVEMENT OF THE NITROGENASE ACTIVITY IN *E. COLI* THAT EXPRESSES THE NITROGEN FIXATION-RELATED GENES FROM *AZOTOBACTER VINELANDII*

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Transfer of *nif* genes from diazotrophs to nondiazotrophic hosts is of increasing interest to engineer nitrogen fixation. The *E. coli* KT strain that expresses the 18 genes (*nifHDKBUSVQENXYWZMF, iscA* and *nafU*) from *Azotobacter vinelandii nif* island had been shown to exhibit the nitrogenase activity [1]. However, the nitrogenase activity of KT strain was still considerably low compared to that of *A. vinelandii*. In this study, we examined the effects of genes possibly involved in electron transfer to NifH on improvement of the nitrogenase activity. Although the introduction of the *nifHDKB* and *nafU* genes (gene group A) into the *fliK* gene locus of KT strain did not affect the nitrogenase activity, the simultaneous introduction of *nifUS* and *iscA* genes, which are involved in the formation of iron-sulfur clusters incorporated into NifH, with gene group A increased the activity. The deletion and overexpression of the pyruvate-flavodoxin oxidoreductase gene (*ydbK*) of KT strain led to abolishment and enhancement of the nitrogenase activity, respectively. Furthermore, the co-overexpression of the flavodoxin gene (*fldA*) with *ydbK* resulted in a further increased activity, compared to that of the KT strain overexpressing *ydbK*. These results suggest that enhanced electron transfer to NifH is an important factor for the improved nitrogenase activity.

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TOWARDS THE MINIMAL N_2-FIXING SYMBIOTIC GENE SET OF THE pSYMB MEGAPLASMID IN THE HOST LEGUME SYMBIONT *SINORHIZOBIUM MELILOTI*

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An ever-burgeoning population requires heightened food production at the cost of sustainable fertilizer practices, leading to systemic nitrogen pollution. Biologically derived reactive nitrogen accounted for the majority of terrestrially fixed nitrogen before the advent of the Haber-Bosch process. The bacteria responsible represent a sustainable source of endogenous reactive nitrogen; understanding the genes involved has significant industrial applications. *Sinorhizobium meliloti* is an important model organism for understanding the ecologically and agriculturally important N_2-fixing symbiosis it forms with legumes. The *S. meliloti* genome consists of a 3654 kb chromosome and two megaplasmids: pSymA (1354 kb) and pSymB (1683 kb). Recent work established a minimal N_2-fixing gene set for the pSymA megaplasmid that is involved directly in the establishment of the nodule and nitrogen fixation.[1] pSymB contains several clusters of genes necessary for symbiotic nitrogen fixation (SNF), some of which are less direct and poorly understood relative to those of pSymA. Deletion analyses identified three regions on pSymB (12% of genome) that were necessary for effective symbiosis with *Medicago sativa* (alfalfa).[2] However, when these regions were combined, they were not sufficient for the formation of effective symbiosis with alfalfa. To establish what was missing, a Cre-loxP strategy was employed to facilitate the construction of numerous, cumulative large deletions of remaining pSymB regions. The less-direct role of pSymB in symbiosis was highlighted by the difficulty in making deletions that did not impact SNF. Moreover, subsequent attempts to identify symbiotic loci of importance in these regions proved challenging. Continued pSymB reduction generated a strain with 363 kb (22%) of the pSymB genome that is 37% as effective as the wild type. Half of the remaining DNA contains no canonically known genes for SNF yet has proven difficult to minimize without incurring significant symbiotic penalty.

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A HIGH-THROUGHPUT PLATFORM FOR NITROGENASE ENGINEERING IN PLANTS

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Engineering plants with the ability to fix N_2 requires the transfer of prokaryotic nitrogen fixation (*nif*) genes. Previous work has shown that homolog mining, involving solubility screenings of different Nif protein variants followed by protein activity assays, is a viable strategy to overcome Nif protein solubility issues and to provide new sources of nitrogenase components with superior properties for expression in plants[1,2]. However, homolog mining is currently labour-intensive and suffers from lengthy protocols for the generation of stable plant transformants. We therefore developed a series of tools to streamline the homolog mining workflow in plant cells. First, we created an informatics tool to facilitate the generation of *nif* gene libraries by automating the fetching of crucial ecology information on the source organisms such as growth temperatures from different online databases. Furthermore, we simplified a high-throughput method for transient expression[3] and adapted it for Arabidopsis suspension cells. We also improved the efficiency of stable transformation, allowing substantially shorter times for obtaining transgenic suspension cell lines and providing a scalable and highly tractable culture system that is well-suited for the needs of plant nitrogenase research. Using this method, we obtained cell lines producing soluble NifU, NifS, FdxN, NifX, and NifB, which is a prerequisite to optimize *in-planta* biosynthesis of NifB-co, a crucial precursor of the essential nitrogenase cofactor FeMo-co.

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IMPROVING THE SOLUBILITY, ABUNDANCE, AND ACTIVITY OF ENGINEERED NIFH AND ANFH IN PLANT MITOCHONDRIA

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Engineering non-legume plants that can fix nitrogen to reduce the need for synthetic nitrogen fertilizer has been a long-standing goal in biotechnology. This could be achieved by transferring the genes that code for nitrogenase - the enzyme that catalyses nitrogen fixation – into plants. There are three different classes of nitrogenase, MoFe-, VFe- and FeFe-nitrogenase, named after the metal present in the catalytic cofactor where N^2 is reduced to NH_3. Nitrogenase and many of its assembly proteins containing metalloclusters are extremely oxygen sensitive, which has been considered a bottleneck to reconstituting activity in plants, Our lab is working on the assembly of MoFe and FeFe nitrogenase in plant mitochondria. This organelle consumes oxygen and contains enzymes involved in metallocluster synthesis and is thus potentially suitable for nitrogenase assembly and activity.

Aside from the known challenges of oxygen and metallocluster assembly, another emerging obstacle for progress has been the low solubility of certain nitrogenase proteins when expressed in plant mitochondria. Past studies have previously expressed numerous proteins of the nitrogen fixation pathway in plant mitochondria [1-3] and found that the key component NifH from the model diazotroph *Klebsiella oxytoca* (Ko) was insoluble. FeFe nitrogenase is considered less active than the MoFe counterpart but is simpler in terms of its biosynthetic components. We have also successfully expressed *Azotobacter vinelandii* (Av) AnfH, AnfD, AnfK, and AnfG in plant mitochondria [Johnston et al., 2023 manuscript in prep]. However, AvAnfH was only partially soluble in this organelle, which could limit FeFe nitrogenase biogenesis and activity. Here we show our progress for improving these features of both KoNifH and AvAnfH when expressed and purified form plant mitochondria using protein engineering approaches.

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SINORHIZOBIUM MELILOTI CONTAINS A FUNCTIONAL PYROPHOSPHATE DEPENDENT PHOSPHOFRUCTOKINASE THAT PLAYS A ROLE DURING SYMBIOTIC DEVELOPMENT

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Central carbon pathways are generally well understood in *S. meliloti*. The catabolism of hexoses occurs using the Entner Doudoroff pathway, and the upper part of the Embden Meyerhof-Parnas pathway is used in a gluconeogenic manner because of the lack of a gene encoding an ATP dependent phosphofructokinase. Here, we show that *S. meliloti* contains a pyrophosphate dependent phosphofructokinse (Pfp). Over-expression of this open reading frame and isolation of the encoded protein showed that it had Pfp activity that could be assayed in both the forward and reverse directions. The activity of this enzyme was also negatively affected by GTP. Surprisingly, *S. meliloti* carrying a mutation in *pfp* did not have any discernable growth phenotypes on defined medium containing either glucose or succinate. In-Seq mutagenesis of the *pfp* mutant strain suggested that growth of this strain on succinate was dependent on the presence of transaldolase (*tal*) and *Smc00535*. Phylogenetic analysis revealed that *pfp* is conserved across the *alpha*, *beta*, and *gamma Proteobacteria*. To corroborate this the genes annotated as *pfp* from a few type strains were codon optimized and able to complement a *pfp tal* lesion in *S. melilolti*. Similarly the *Smc00535* gene was also over-expressed and mutated. SMc00535 was shown to have F-1,6-biP phosphatase activity. Whereas a mutation in SMc00535 could grow on defined medium containing either glucose or succinate, a strain carrying mutations in both *pfp* and *SMc00535* was unable to grow using gluconeogenic substrates. Symbiotic testing of the mutants showed that whereas a strain carrying only a *pfp*, tal, or SMc00535 mutation did not affect overall nitrogen fixation, a strain carrying both a *pfp* and a *tal* mutation resulted in a decrease of nitrogen, whereas a strain carrying both *pfp* and *SMc00535* resulted in an inability to fix nitrogen as measured by dry weight accumulation.

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ANFO CONTROLS THE FIDELITY OF FE-ONLY NITROGENASE DURING THE MATURATION PROCESS

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The aerobic free-living diazotroph *Azotobacter vinelandii* produces three genetically distinct but functionally and mechanistically similar nitrogenase isoforms[1–3]. These isoforms are designated as Mo-dependent, V-dependent or Fe-only nitrogenases based on the metal composition of their associated active site cofactors[4]. In previous work, we found that AnfO preserves the fidelity of the Fe-only nitrogenase by preventing the misincorporation of FeV-cofactor, which is formed when V is present in the growth media even at trace levels[5]. In this work, we show that the role AnfO is not exclusive for FeV-cofactor but instead, also prevents misincorporation of FeMo-cofactor when even trace levels of Mo are available. Phylogenetic comparisons and predicted protein structure reveal that AnfO is a modular protein containing two conserved domains separated by a non-conserved linker domain. We explored the AnfO mechanism and found that the two AnfO domains have distinct functions that allow the protein to interact with an immature form of the Fe-only nitrogenase and to bind FeMo- and FeV- cofactors thereby preventing their misincorporation into Fe-only nitrogenase.

Based on these results, we propose the existence of a novel post-transcriptional/post-translational mechanism that controls the fidelity of the Fe-only nitrogenase by preventing incorporation of the incorrect cofactor into FeFe protein during the maturation process. This work contributes to our understanding of the role of Fe-only nitrogenase-associated proteins and demonstrates AnfO is a nitrogenase catalytic cofactor-binding protein.

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MOLECULAR HOMEOSTASIS OF CARBON AND NITROGEN METABOLISMS IN *NOSTOC SP.* PCC 7120

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Diazotrophic cyanobacteria fix both atmospheric carbon (C) and nitrogen (N) into biomass and contribute to the bioavailability of both chemical elements in the biosphere. This ability also brings these phototrophic prokaryotes at the forefront of many biotechnological applications (i.e. sustainable production of chemicals and fertilizers). Understanding the metabolism of these organisms is therefore seminal to both predicting perturbations of natural ecosystems' functionality, as well as developing robust biotechnological solutions for a sustainable global economy.

In this work, we investigated the molecular homeostasis of C and N metabolisms in the model experimental system *Nostoc sp. PCC 7120*. Both genetic (i.e. *Δamt* mutation) and environmental treatments (i.e. different N sources) led to a complex metabolic remodelling, suggesting C and N metabolism must be functionally coordinated to achieve homeostasis and AMT transporters were suggested to be integral players of both metabolic networks, bridging signalling with metabolic regulation, via a molecular transduction cascade, likely triggered by the PII protein. Our data also suggest the existence of a central regulatory/signalling bottleneck controlling the internal supply of N to the cell, through a similar riboswitch mechanism as reported in *Synechocystis sp.* PCC 6803.

To investigate further the existence of this mechanism, we generated a collection of mutants in which the functionality of the main entry node of N in the metabolism (i.e. GLUTAMINE SYNTHETASE, GS) is genetically controlled. We i) overexpressed IF7A, a small peptide that acts as post-translational negative regulator of GS activity, under the control of an inducible promoter and ii) Knocked-out the small RNA *nsiR4* that blocks the transcription of the *gifA* gene, coding for IF7A. The two complementary approaches were designed to reduce the functionality of GS and highlight the role of additional regulatory mechanisms on N metabolism homeostasis. Targeted quantification of key proteins and enzyme activities highlight the existence of further regulatory mechanisms indeed that controls the internal N status of the cell.

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THE ABILITY TO UTILIZE GLUCOSE AND FRUCTOSE IMPROVES ROOT COLONIZATION AND PLANT GROWTH PROMOTION BY AZOSPIRILLUM BRASILENSE SP7

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Azospirillum brasilense is a plant growth promoting rhizobacterium (PGPRs), which is an efficient colonizer of the rhizosphere of a large number of leguminous and non-leguminous plants. An important reason for its rhizocompetence is its ability to preferentially use dicarboxylates for growth and nitrogen fixation. However, its growth is restricted on carbohydrates, such as D-glucose, D-mannose and sucrose but it grows on D-fructose, D-galactose and L-arabinose. But, its close relative *A. lipoferum* 4B grows well on D-glucose. By comparing the genomes of both the species, the genes of *A. lipoferum* 4B responsible for conferring D-glucose utilization ability in *A. brasilense* Sp7, were identified by cloning them individually or in combination in a broad host range expression vector, mobilizing them in *A. brasilense* Sp7 and examining the ability of exconjugants to use D-glucose as sole source of carbon for growth. Constitutive expression of the 5 genes of *A. lipoferum* 4B encoding glucose-6-phosphate dehydrogenase (G6PD) and 4 components of glucose phosphotransferase system (EIIA, EIIBC, NagA & GFPT) via broad host range expression vector improved D-glucose utilization ability of *A. brasilense* Sp7. Further, inoculation of rice seedlings with the GFP-tagged, glucose-utilizing engineered strain of *A. brasilense* Sp7 showed significant improvement in the root colonization and shoot biomass of rice seedlings [1]. Although fructose is a less preferred carbon source for *A. brasilense* Sp7 for growth, it is able to induce the expression of proteins involved in making a Type 6 Secretion System (T6SS), which is able to kill *E.coli* and other bacteria in a contact-dependent mode. Analysis of the *A. brasilense* Sp7 genome showed that it encodes two gene clusters which are homologous to fructose-phosphotransferase system (PTS^{Fru}). Inactivaion of one of the two PTS^{Fru} led to the complete loss of growth on fructose as carbon source. It appears that in the absence of preferred sources of carbon, *A. brasiliense* Sp7 uses T6SS to compete with other microbes and survive in the rhizosphere

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GLUTAREDOXIN5 IS REQUIRED FOR AN OPTIMAL NITROGENASE ACTIVITY

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Iron metabolism is central to nitrogenase activity, as this enzyme relies on three different iron-sulfur clusters to catalyze the reduction of N_2 into NH_3. The diazotrophic bacterium *Azotobacter vinelandii* usually uptakes iron via FeoB and then distributes it among different iron-containing proteins. Two of the main hubs of iron trafficking are bacterioferritin (BfrA), the main cytosolic iron storage protein, and NifU, the scaffold protein where the Fe-S clusters used by nitrogenase are first assembled (1). To identify new elements in the cytosolic iron trafficking network of *A. vinelandii*, we performed pull-down assays using purified NifU and BfrA proteins as bait for *Azotobacter vinelandii* DJ extracts. In these assays, glutarredoxin5 (Grx5) was pulled down with both bait proteins.

Interaction assays between purified NifU, BfrA and Grx5 proteins were carried out to confirm the pull-down results. Grx5 and NifU always interacted, independently of their respective iron-loading states. However, BfrA and Grx5 only interacted when BfrA was iron loaded and Grx5 was in its iron-deficient form, indicating a possible iron transfer from BfrA to Grx5. Mutants with chromosomal deletions of *grx5* or *bfrA genes* fixed significantly less N_2 than the wild type, evidencing that these proteins are required for optimal nitrogenase activity. The double *grx5 bfrA* mutant showed similar nitrogenase activity to the single mutants, suggesting that BfrA and Grx5 are part of the same pathway.

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EXPLORING THE ROLE OF TWO RpoN IN BRADYRHIZOBIUM SP. DOA9 IN SYMBIOSIS AND FREE-LIVING GROWTH USING SYNCHROTRON FTIR MICROSPECTROSCOPY

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The *Bradyrhizobium* sp. DOA9 strain contains a chromosomal (c) and plasmid (p) encoded RpoN proteins. Normally, RpoN in bacteria has diverse physiological functions. In rhizobia, RpoN plays a key role in the transcription of nitrogen fixation (*nif*) genes. Based on the single and double *rpoN* mutation and construction of reporter strains, it was observed that the inactivation of *rpoNc* or *rpoNp* severely impacts the physiology of the bacteria under free-living conditions, such as the bacterial motility, carbon and nitrogen utilization profiles, exopolysaccharide (EPS) production, and biofilm formation. However, free-living nitrogen fixation appears to be under the primary control of RpoNc. Interestingly, drastic effects of *rpoNc* and *rpoNp* mutations were also observed during symbiosis with *Aeschynomene americana*[1]. This may be related to the low production of cellular surface polysaccharides (CSP) of the mutants. Analysis of CSP from the *rpoN* mutants using FTIR revealed the absence of certain components such as lipid, carboxylic group, polysaccharides-pyranose ring, and β-galactopyranosyl residues. The chemical distribution map of the DOA9WT nodule indicated a high consistency of lipid, protein, and carbohydrate across the nodule compared to the small and less intense red-colored nodules of the *rpoN* double mutation strain. These data confirm the role of the two *rpoN* in *Bradyrhizobium* strain DOA9.

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P 106

FABRICATION OF CELL PLASTICS AS NOVEL CARBON NEUTRAL MATERIALS

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Nitrogen fixation is an important technology that utilizes the atmosphere as a resource to produce useful materials. Algae produce peptides via nitrogen fixation, and in this process, carbon dioxide is used as a carbon source, thereby significantly contributing to the reduction of greenhouse gas emissions. For the practical use of these technologies, it is important to develop novel technologies to produce materials from the algae in an industrial scale.

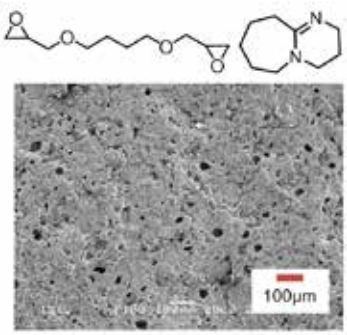

Figure 1 SEM image of the cross section of the cell plastic

By 2020, 367 million tons of plastic had been produced annually worldwide, creating serious problems such as increased microplastics and greenhouse gas emissions. Various biodegradable resins and biopolymers have been developed to reduce carbon emissions. However, these are not sustainable technologies because they consume enormous amounts of energy in the process of fermenting plants and extracting useful chemical components. Furthermore, they do not provide sufficient resources to meet the global demand for plastic production.

We developed a technology to directly convert green algae cells into resin. Green algae cells grow by absorbing carbon dioxide from the atmosphere, and the cells were directly turned into a resin to obtain “cell plastics” without a process to extract the components from the cells. Resins were obtained by mixing chlorella and 1,4-butanediol diglycidyl ether in the presence of catalytic amount of a base. When 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) was used as the base, Young’s modulus and tensile stress were 1.29 GPa and 12.8 MPa, respectively. The bio cell content was 80% (Figure 1), and the 10% weight loss temperature of the resin was 262 °C. In addtion, films from the cell plastics were fabricated using biodegradable and water-soluble polyvinyl alcohol (PVA) as a matrix, in which a cell-to-matrix effect thorough hydrogen bonds enhanced the mechanical properties.[1]

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MANUFACTURING L-GLUTAMATE FROM AERIAL NITROGEN USING NITROGEN-FIXING *KLEBSIELLA OXYTOCA*

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L-Glutamate (Glu), one of the most biologically essential compounds, is worldwide produced for various practical uses such as pharmaceuticals, clinical nutrients, and umami seasoning. The current Glu production requires a large amount of industrially produced ammonium; hence, the development of bioeconomical Glu-producing processes has long been awaited. Here, we demonstrate a novel strategy for Glu production from aerial nitrogen using *Klebsiella oxytoca* NG13[1], a diazotroph isolated form the rhizosphere of rice. First, we investigated the carbon sources to enhance the nitrogenase activity of NG13 under the atmospheric condition. The combination of glucose and citrate resulted in three-fold higher activity than glucose as a sole carbon source, but NG13 did not excrete Glu in this condition. Next, we genetically engineered NG13 in two ways to increase the flux to 2-oxoglutarate, a direct precursor of Glu synthesis. Under glucose-citrate-coexisting conditions, overproduction of either citrate synthase of *Corynebacterium glutamicum* (CgCS) or Na^+-dependent citrate transporter (CitS) of NG13 led to extracellular Glu production of 109 or 19 mg/L, whose carbon origin was glucose or citrate, respectively. The nitrogen origin of Glu was confirmed to be aerial nitrogen by $^{15}N_2$ incorporation assay. A combination of CgCS and CitS overproduction led to seemingly additive Glu production of 153 mg/L, interestingly, the carbon origin of Glu was only citrate, suggesting the carbon flow for Glu synthesis is drastically altered. Lastly, we further improved the culture conditions considering the oxygen supply, resulting the extracellular Glu production was over 1 g/L.

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P 108

THE MBFA IRON EXPORTER SAFEGUARD RHIZOBIA FROM AN IRON OVERLOAD BY THE HOST LEGUME

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During the rhizobia-legumes symbiosis iron (Fe) stands out as an essential cofactor in keystone and conspicuous metalloenzymes such as the nitrogenase, cytochromes oxidases or the leghaemoglobin. Legumes provides this nutrient to the nitrogen-fixing rhizobia by a dedicated set of transporters. First, the plant host diverts iron to the nodule from the root vasculature, incorporate it on the infected cells, and then deliver it to the symbiosome, the organelle-like structure where the endosymbiotic bacteria reside [1,2]. By this system, the concentration of iron within the symbiosomal space increases to an estimated 0.5-2 mM range [3], which would result toxic for most free-living bacteria [4].

First, we determined the iron content in nodule bacteria and free-living grown cells, observing that was always higher in bacteroids. Interestingly, *Sinorhizobium meliloti* 2011 (Sm2011) cells isolated from *Medicago sativa* nodules have five times more iron that any other nodule-rhizobia. How endosymbiotic bacteria tolerate those concentrations? Mining of previous transcriptomes in Sm2011 and *Rhizobium leguminosarum viciae* bv. 3841 (Rlv3841) revealed a high expression of the iron exporter Membrane Bound Ferritin A (MbfA), a member of the Ccc1/VIT1 transporter superfamily. *MbfA* is upregulated by iron in free-living conditions, and in the apical part of the nodule. This gene is responsible for conferring tolerance to ferrous toxicity and ROS in free living conditions in various rhizobia strains, but essential only for the symbiosis between Sm2011 and *Medicago sp.*

Summarizing, MbfA expression would safeguard rhizobia from the risk of being overload with iron by the legume host. Since that seems not to be the case in most legume hosts, we are currently testing whether manipulating the iron content of rhizobia within the nodule by expressing various iron transporters would increase its nitrogen fixation capacity, and result in higher plant yields.

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P 109

FUNCTIONAL ANALYSIS OF A HOST-SPECIFIC DIAMINOBUTYRATE AMINOTRANSFERASE FROM *RHIZOBIUM LEGUMINOSARUM*

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Rhizobium leguminosarum bv. viciae (*Rlv*) is an α-proteobacterium able to induce nitrogen-fixing root nodules in association with *Vicia, Pisum*, *Lens* and *Lathyrus*. Although the mechanisms governing the exchange of signals and nutrients between *Rhizobium* and legume plants have been subject to detailed studies, little is known about the differences in the environment provided to a given microsymbiont by different hosts and how rhizobia adapt to them. Comparative proteomic analysis of bacteroids induced by *Rlv* UPM791 strain in lentil and pea plants showed a significant number of proteins with host-dependent expression [1]. Among them, a protein (C189) overexpressed in pea bacteroids was identified. This protein, encoded by gene *rlv_189* located in the symbiotic plasmid, is similar to a diaminobutyrate-2-oxoglutarate aminotransferase (DABA-AT).

DABA-AT activity was demonstrated in *Rlv* UPM791 cell extracts and with purified C189 protein. The *rlv_189* gene was strongly induced in the central, active area of pea nodules, but not in lentil. A mutant defective in C189 was impaired in growth with L-homoserine as N source and also in symbiotic performance and nodulation competitiveness in association with pea plants. Metabolomic profiling showed significant differences among the wild type and *c189*-deleted mutant strains in pea bacteroids. These experiments also revealed that the DABA-AT reaction was oriented towards the production of 2,4-diaminobutyrate (DABA) in pea nodules. The mutant showed reduced levels of the pantothenate precursor β-alanine in pea bacteroids. Inclusion of DABA or L-Hse as N source suppressed pantothenate auxotropy of the strain, suggesting DABA as potential source for this relevant growth factor. These data indicate that *Rlv* UPM791 C189 enzyme is part of an adaptation mechanism of this bacterium to a homoserine-rich environment such as pea nodule and rhizosphere.

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NEGATIVE REGULATION OF SYMBIOTIC NITROGEN FIXATION BY A DEFENCE-RELATED MOLECULAR MECHANISM

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Legumes accommodate symbiotic rhizobacteria within plant cells of special organs, the root nodules, where rhizobia bind elementary nitrogen from the air. As a result of this symbiosis, cultivated legumes are able to provide themselves and subsequent rotation crops with nitrogen, reducing requirements for environmentally and economically costly mineral nitrogen fertilization. The past two decades of research on *Rhizobium*-legume interactions have provided unique insights into genetics and molecular biology of nodulation[1]. However, relatively little is known about the genetic mechanisms controlling or limiting the actual nitrogen fixation and symbiotic efficiency. Plants have evolved diverse strategies to limit microbes through transcriptional activation of antimicrobial molecular mechanisms. It is conceivable that genes supporting such microbial control have been co-opted to control bacterial symbiosis[2]. We hypothesized that negative regulators of symbiosis may be derived from ancestral defence genes through gene duplication and subsequent neo-functionalisation. Screening transcript levels of *Medicago truncatula* defence associated genes during the symbiosis with *Sinorhizobium meliloti* we indentified the symbiosis-specific upregulation of the β-*Glucan-Binding Protein 1* (*GBP1*) gene. *GBP* genes encode dual domain proteins with glucan-binding and hydrolytic activities towards microbial β-1,3/1,6-glucans[3, 4]. The Medicago genome encodes twelve members of the GBP family. Most of them are upregulated upon challenge with the pathogenic fungi and oomycete or by treatment with pathogen-associated molecular patterns such as flagelin or laminarin (a branched glucan, structurally similar to glucans from cell walls of filamentous pathogens). More detailed expression analysis shows that symbiotic specific induction of the *GBP1* gene is dependent on Nod factor perception and NIN-mediated transcription regulation. This suggests that activation of *GBP1* during interactions with rhizobia is a part of the host symbiotic program. Genetic deregulation of *GBP1* expression showed that GBP1 negatively regulates nitrogen fixation depending on the microsymbiont efficiency. More over, inactivation of GBP1 increases nitrogen fixation without affecting nodule numbers providing inroads for engineering legumes with increased productivity for sustainable nitrogen provision.

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P 111

CATCHING RHIZOBIA TO INTRODUCE HIGH PROTEIN CONTAINING SOYBEAN FOR A SUSTAINABLE AGRICULTURE IN EUROPE

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To develop sustainable protein products, Europe would strongly benefit from soybean production at northern latitudes. However, soybean is not adapted to these environmental conditions and therefore the cultivation of protein-rich soybean is challenging. While several soybean varieties have been bred for optimal growth, to guarantee consistent high-protein rich beans, plants also need effective interaction with suitable soil bacteria that can fix nitrogen in root nodules. These nitrogen-fixing bacteria allow legumes to act as natural nitrogen fertilizers and green indicators of soil nutrition, and as such improve crop yield without the damaging effects of chemical nitrogen fertilization. Likewise, legumes contribute to solving major environmental challenges such as nitrogen pollution and declining soil quality. The current commercial inoculants are not adapted to cultivation under the local soil and environmental conditions of North-Western Europe. This hampers the interaction and leads to insufficient bean protein content for human food consumption. Local strains, adapted to our conditions may be more competitive and have a superior positive effect on soybean production. We set up a citizen science project to trap endogenous nitrogen-fixing bacteria that nodulate locally grown soybean. To have access to a large geographical gradient and different soil types in Flanders, over one thousand citizens across Flanders were recruited to grow and monitor soy plants, frequently entering data regarding plant phenotype into a platform specifically designed for the project. The outcome of the project in terms of microbial discovery but also correlations between soil parameters such as nutrient levels, soil texture, and nodulation performance will be discussed.

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IDENTIFICATION OF *MEDICAGO TRUNCATULA* NCR PEPTIDES CRUCIAL OR NON-ESSENTIAL FOR SYMBIOTIC NITROGEN FIXATION

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In the nodules of Inverted Repeat-Lacking Clade legumes, including *M. truncatula*, nitrogen-fixing rhizobia undergo terminal differentiation resulting in elongated and endoreduplicated bacteroids specialised for nitrogen fixation. This irreversible transition of rhizobia is mediated by host produced nodule-specific cysteine-rich (NCR) peptides, of which about 700 are encoded in the *M. truncatula* genome. The high number of *NCR* genes implied that the function of NCR peptides might overlap or partially overlap and the NCRs act redundantly. Recent studies revealed that some NCR peptides, NCR169, NCR211 and NCR247 are essential for terminal bacteroid differentiation and persistence, and the mutant plants defective in these genes form ineffective symbiotic nodules. We have investigated the symbiotic role of several NCR peptides using forward and reverse genetic approach. We have analysed the phenotype of ineffective symbiotic mutants of *M. truncatula* and identified three additional NCR peptides crucial for nitrogen-fixing symbiosis. We have carried out the targeted mutagenesis of selected *NCR* genes using an *Agrobacterium rhizogenes*-mediated CRISPR-Cas9 system and identified several NCR peptides non-essential for symbiosis between *M. truncatula* and the studied rhizobia strains. We have regenerated mutant plants from gene edited hairy-roots for detailed phenotypic characterization. The identification of novel crucial NCRs implies that further forward and reverse genetic studies might extend the cluster of universal and essential NCR peptides of *M. truncatula*.

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POLYAMINES ARE ESSENTIAL FOR BACTEROID MAINTENANCE AND N_2-FIXATION

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Polyamines are ubiquitous positively-charged molecules involved in a variety of physiological processes in all domains of life. The most basic polyamines are diamines such as cadaverine or putrescine, which are derived from amino acid catabolism. Putrescine is the precursor for more complex polyamines such as homospermidine or spermidine. Polyamines are involved in ribosome (30S subunit) assembly and translational activity. In addition, they are involved in mediation of oxidative, osmotic, and acidic stress.

Although comprehensive knowledge about the exact mechanisms involved is still missing, polyamines are also important in host – pathogen associations, including in animal- and plant-pathogenic bacteria. However, comparatively little is known about the importance of polyamines in rhizobia – legumes symbioses.

Here, we describe the importance of “Homospermidine Synthase” (Hss) in *Rhizobium leguminosarum* bv. *viciae* 3841 and other rhizobia. Importantly, for most rhizobia, homospermidine is the only complex polyamine produced and appears to have functions that cannot be covered by putrescine. Mutants in *hss* were reduced in growth and showed an increased susceptibility to stress such as acid shock. Moreover, in symbiosis with host plants, the mutants displayed reduced nitrogen fixation and smaller nodules. Over prolonged growth periods this resulted in stunted and chlorotic plants. Detailed morphological analysis showed signs of premature senescence and bacteroid degradation. Heterologous complementation experiments demonstrated that the role of homospermidine in the rhizobia – legumes symbioses is not unique as it can be replaced with other complex polyamines. RNAseq analysis of the mutant revealed a drastically changed transcriptional landscape compared to the wild type.

Overall, we show that complex polyamines are needed to maintain a normal physiology, adapt to stressful environments, and for maintaining specifically bacteroid functioning and integrity.

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FUNCTIONAL AND REGULATORY ANALYSIS OF A HOST-DEPENDENT ABC METAL TRANSPORTER SYSTEM FROM *RHIZOBIUM LEGUMINOSARUM*

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Nitrogen-fixing *Rhizobium*-legume symbiosis constitutes an alternative to the use of nitrogen fertilizers. The establishment of the symbiosis requires sophisticated plant- and bacteria-dependent mechanisms that lead to the formation of nodules where rhizobia differentiate into bacteroids, the symbiotic nitrogen-fixing form. Proteomic analysis of lentil and pea nodules induced by *Rhizobium leguminosarum* bv. viciae UPM791 revealed that over 100 proteins show a host-dependent expression[1]. Among these proteins, we identified a pea-overexpressed metal binding protein (RLV_3444), a component of the ABC transporter system RLV_3442-3444. The overexpression of this protein in peas suggests that the provision of some metal(s) to the bacteroid is more restrictive in this host. This work aims to study the functional role of the RLV_3442-3444 metal transporter system in the *Rhizobium*-legume symbiosis.

Results have shown that zinc concentration in pea bacteroids induced by a mutant strain affected in the transporter system decreased in comparison with the wild type. Functional analysis of RLV_3442-3444 has revealed that RLV_3444 replaces the role of ZnuA, the metal-binding protein of the high-affinity zinc transport system ZnuABC, under free-living and symbiotic conditions. The defective growth phenotype of the RLV_3444/ZnuA mutant strain under zinc-depleted conditions was complemented by supplementation with zinc, but not with manganese or iron. Transcriptional fusions to the reporter gene *gusA* have shown that the system is expressed under zinc-limiting conditions and repressed in the presence of this metal in a transcriptional regulator Zur-dependent manner.

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INVESTIGATING THE ROLE OF THE PLANT CIRCADIAN CLOCK IN REGULATING THE *MEDICAGO TRUNCATULA* – RHIZOBIA SYMBIOSIS VIA NODULE-SPECIFIC CYSTEINE-RICH PEPTIDES

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An established legume-rhizobia symbiosis results in a mutualistic relationship whereby the plant host receives fixed-nitrogen from the bacteria in exchange for dicarboxylic acids. This plant-microbe interaction is highly complex, requiring the regulation of multiple metabolic and physiological processes in both the host and symbiont. Nodule-specific cysteine rich (NCR) peptides are a family of plant defensin-like peptides exclusively expressed within rhizobia-inoculated indeterminate nodules of inverted-repeat lacking clade (IRLC) legumes. NCRs have known functions in regulating rhizobia differentiation into nitrogen-fixing bacteroids, and new evidence from our lab suggests that NCR peptides have additional diverse regulatory roles in regulating plant-microbe interactions, including in nodule development. Recent work has uncovered circadian control of NCR expression within nodules; we hypothesise that the plant circadian clock regulated plant-microbe interactions within nodules through the expression of NCRs. This research project is investigating the relationship between the plant circadian clock and nodulation via NCRs using the model system *Medicago truncatula* and its symbiont *Sinorhizobium medicae.* We are generating NCR promoter: luciferase reporter vectors for the 45 rhythmically expressed NCRs and assessing how their expression varies when 1) *M. truncatula* is in symbiosis with different rhizobial strains and 2) in different day/night cycles or constant light conditions. By also manipulating NCRs to abrogate their rhythmicity this work is helping to characterise the role of NCRs in mediating circadian effects on nodulation.

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FURTHER CHARACTERIZATION OF THREE *L. JAPONICUS* NITRATE TRANSPORTER GENES AND THEIR INVOLVEMENT IN NODULE FUNCTIONING

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Genes encoding for nitrate transporters of both NPF and NRT2 families are largely represented in the subcategory of nodule induced genes identified in mature N_2-fixing nodules. We have recently functionally characterized three *L. japonicus* nitrate transporters, *LjNPF8.6, LjNPF3.1* and *LjNRT2.4*. These three genes play positive roles in the control of nitrate flux through cytosolic and symbiosome compartments of mature *Lotus* nodules [1-3]. This route of nitrate appears to be particularly important under hypoxic conditions when a nitrate-dependent respiratory chain must be achieved to support the energy status for an efficient nodule functioning [2]. NPF transporters may own a peculiar dual transport capacity as some of them, display the ability to transport both nitrate and hormones. We present a further characterization of the *LjNPF3.1* gene by analyzing its involvement in gibberellin-related pathways affecting the nodule functioning. Furthermore, in order to better investigate the combined action of the three transporters we have obtained double mutants with different combination of knock out genetic backgrounds. Results of the phenotypic characterization of *npf8.6-npf3.1* and *npf8.6-nrt2.4* double mutants will be presented.

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POSTER
Nodule Development

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UNRAVELING THE ROLE OF NODULE-SPECIFIC GRPs IN NITROGEN-FIXING SYMBIOSIS

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In order to understand the molecular mechanisms leading to symbiotic nitrogen-fixing root nodules in legumes, many studies were performed to identify nodule-specific genes and gene families. Among them two nodule-specific gene families encode secreted peptides, namely glycine-rich proteins (nodGRPs) [1] and the cysteine-rich peptides (NCRs) [2]. These symbiotic peptides have evolved exclusively in the IRLC legumes, and ~700 NCR genes and 34 GRP genes were identified in the *Medicago truncatula* genome that are active at different stages of symbiotic nodule development[2]. While the role of NCRs in the terminal differentiation of bacteroids in nitrogen fixing root nodules has been demonstrated, little is known about the possible symbiotic functions of the nodGRP peptides. GRPs have been described in a wide variety of plant species with a glycine content of 80% ordered in typical repeated motifs, that perform variable roles often in biotic and abiotic interactions between plants and their environment[3]. The nodGRPs are shorter and contain less glycines, without clear motif structures and only produced during root nodule development[4].

Our aim in this work is to understand the symbiotic function of two members of the *MtnodGRP* family by using different experimental approaches. We have been analysing the symbiotic phenotypes of the *MtnodGRP* overexpressing and mutant *Medicago* lines and studying the regulation of *nodGRP* genes. Moreover, we have been generating and using constructs of *MtnodGRP* genes encoding tagged recombinant proteins to help the identification of interacting partners of the nodGRPs and their localization in the symbiotic nodule cells.

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REGULATOR OF SYMBIOSOME DIFFERENTIATION (RSD) MEDIATED TRANSCRIPTIONAL CONTROL OF *MEDICAGO TRUNCATULA* NODULE DEVELOPMENT

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The intracellular accommodation of rhizobia in leguminous plants results in the formation of a unique organelle-like structure called the “symbiosome”. Symbiosome development and differentiation are highly regulated. One of the key transcriptional regulators involved in symbiosome differentiation is the “Regulator of Symbiosome Differentiation” (RSD), which is a transcription factor containing a C_2H_2 DNA binding domain and an ERF-associated Amphiphilic Repression (EAR) domain.
Previously, we have shown that in *rsd-1* plants, symbiosomes cannot complete their differentiation and degrade in the invasion zone, resulting in a “fix-” phenotype[1]. To gain a better understanding of the role of RSD in symbiosis, we have studied the activity of the *RSD* promoter using a GUS assay, which confirmed its expression exclusively in the invasion and Interzone zones of *Medicago truncatula* nodules, with maximum expression at the distal invasion zone. RSD-GFP fusion protein localizes to the nucleus of the infected cells of the invasion and interzone. The *pRSD* contains five IPD3/Cyclops recognition sequences (CYC-RE). Transactivation assay and truncated promoter-driven GUS assay confirm that IPD3 activates *RSD*. RSD locus contains a long non-coding RNA we named it *DSR* (1054 bp) that is present in the opposite strand of RSD. Promoter activity of *DSR* was found to be restricted to the meristematic zone of R108 nodules, while *RSD* expression is limited to invasion zone. The transcriptional regulation that restricts *RSD* and *DSR* expression especially has been investigated. To understand the fine transcriptomic regulation during bacteroid differentiation in the invasion zone, translatome profiling using the *pRSD* has been undertaken. Additionally, yeast two-hybrid (Y2H) assays have been employed to identify the RSD interacting proteins. These findings provide a foundation for further elucidation of the interacting transcriptional network of RSD and its regulation in governing symbiosome development.

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TARGETED MUTAGENESIS OF *MEDICAGO TRUNCATULA NODULE-SPECIFIC CYSTEINE-RICH* (*NCR*) GENES USING *AGROBACTERIUM RHIZOGENES*-MEDIATED CRISPR-CAS9 SYSTEM

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Medicago truncatula is a well-established model organism for studying nitrogen fixing symbiosis between Inverted Repeat Lacing Clade (IRLC) legumes and rhizobium bacteria. The genomes of IRLC legumes contain a smaller or larger gene family of nodule-specific cysteine-rich (NCR) peptides which mediate terminal bacteroid differentiation in symbiotic nodules of these legumes[1]. The family of *NCR* genes consists of about 700 members in *M. truncatula* and many of them have strong antimicrobial activity against wide range of bacteria[2]. Although a great collection of deletion and insertion mutants is available for *M. truncatula,* a relatively few mutants defective in *NCR* genes have been isolated. Thus far only 4 NCRs essential for bacteroid development and persistence in *M. truncatula* nodules were identified by exploiting the mutant collections[3,4,5] and an additional one, NCR247 has been identified by reverse genetic approach[6]. In order to study the function of the additional NCRs in nitrogen-fixing symbiosis, we applied the CRISPR-Cas9 gene editing system to analyse the nodulation phenotype of mutant roots mutated in *NCR* genes. We have selected *NCR*s coding for peptides with diverse biochemical properties and targeted them by the CRISPR-Cas9 system using the rapid *Agrobacterium rhizogenes* mediated hairy root transformation. Our results identified NCRs which are not crucial for nitrogen-fixing symbiosis and also proved that higher number of genes can be effectively screened using the optimized gene editing mutagenesis pipeline.

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IDENTIFICATION OF A NOVEL PLAYER REQUIRED FOR INFECTION THREAD PROGRESSION WITHIN NODULES OF *MEDICAGO TRUNCATULA*

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Legume plants, such as *Medicago truncatula*, form a symbiotic relationship with nitrogen-fixing rhizobia which are internalised within macroscopic root structures called nodules[1]. Generation of calcium oscillations in root hairs are a hallmark of the early signalling between rhizobia and their symbiotic legume hosts, which are essential for bacterial infection and the activation of nodule development[2,3]. Rhizobia are delivered to the developing nodule primordia within tubular structures known as infection threads[3] (ITs) that are directed through the cortical tissue before being released as infection droplets into specialised plant cells in the nodule[4]. The indeterminate nodules formed by *M. truncatula* harbour a persistently active apical meristem, giving these nodules a conical shape and forming distinctive zones from the tip to the base of the nodule which represent successive developmental stages for the differentiating rhizobia[5]. Within the infection zone, ITs continuously release bacteria into plant cell[5], which then differentiate into a nitrogen-fixing state. A mutagenesis screen for players in the symbiosis signalling pathway identified a novel mutant with impaired Infection Thread Progression (ITP) in the infection zone of the nodule, which we named *itp-1*. Combining phenotypic characterisation and functional complementation of *itp-1* nodule infection with protein-protein interaction assays, we ascertain the role of ITP-1 in the nodule infection of *Medicago truncatula.*

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TWO MEMBERS OF A NODULE-SPECIFIC CYSTEINE-RICH (NCR) PEPTIDE GENE CLUSTER ARE REQUIRED FOR SYMBIOTIC INTERACTION BETWEEN MEDICAGO TRUNCATULA AND RHIZOBIA

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Legume plants have evolved beneficial interaction with rhizobia that allows them to cope with nitrogen-limited soil conditions. The symbiotic interaction takes place in a special organ, the root nodule, where the bacteria transform to endosymbiotic lifestyle and fix the atmospheric nitrogen to ammonia. The rhizobia partners of the Inverted Repeat-Lacking Clade (IRLC) legumes, including *M. truncatula*, undergo terminal differentiation, resulting in elongated and endoreduplicated bacteroids specialized for nitrogen fixation. A subset of *M. truncatula NCR* genes, *NCR169*, *NCR211*, *NCR247, NCR343*, and *NCR-new35* has been found to be crucial for the differentiation and persistence in the symbiotic nodules. Two other NCRs (NFS1 and NFS2) control the symbiotic interaction in a strain-specific manner.

Here we show that the *M. truncatula* symbiotic mutant, NF-FN9285 is deficient in a cluster of *NCR* genes. We demonstrated that the closely related peptides NCR086 or NCR314 are essential for persistence of differentiated bacteroids of most of the *Sinorhizobium* strains. Despite the difference of the expression level of *NCR086* or *NCR314*, either of them is able to rescue the symbiotic phenotype of mutant NF-FN9285, indicating that the expression level of these *NCR*s is not vital for their biological function.

Our results extend the list of NCR peptides essential for nitrogen-fixing symbiosis between *M. truncatula* and *Sinorhizobium* sp.

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FACTORS GOVERNING ATTACHMENT OF *RHIZOBIUM LEGUMINOSARUM* TO LEGUME ROOTS AT DIFFERENT pHs

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Primary attachment of rhizobia to host legume roots, the first physical interaction during symbiosis, depends on pH. We have used genome-wide insertion sequencing (INSeq), together with luminescence-based attachment assays, and demonstrate that primary attachment of *Rhizobium leguminosarum* biovar *viciae* 3841 to *Pisum sativum* (pea) roots is more complex than was previously thought [1]. In total, 115 genes are needed for initial attachment under one or more of the conditions (acid, neutral or alkaline pH) investigated, with 22 required under all conditions. These include those encoding a cell-surface filamentous hemagglutinin adhesin (RL4382) and its transporter (RL4381), transmembrane protein RL2400, RL3752 (PssA, glycosyl transferase) affecting capsular polysaccharide and transcriptional regulator RL4145 (PckR). The 54 genes required for attachment at pH 7.0 were investigated for the effect mutation has on the ability to form a nitrogen-fixing nodule.

Unsurprisingly, the bacterial cell surface plays a key role in attachment to roots, but environmental conditions influence genetic requirements which are therfore not necessarily constant. For example, glucomannan biosynthesis protein A (GmsA, RL1661) is required to attach to roots at pH 6.5 but not at pH 7.5 [2]. We demonstrate by INSeq and attachment assays using Lux-labelled bacteria a requirement for *gmsA* at pH 6.5. and pH 7.0, while it is not required at pH 7.5.

Our results demonstrate the complexity of primary root attachment and diversity of mechanisms involved in the initial reaction between bacteria and plant roots on their pathway to successful symbiosis.

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HORMONAL PATHWAYS CONTROLLED BY TALE TRANSCRIPTION FACTORS DURING SYMBIOTIC NODULE FORMATION

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Homeodomain transcription factors (TF) play major roles in the development of complex organisms. In higher plants, the TALE (Three Amino acid Loop Extension) superclass of transcription factors is characterized by three extra amino acids between helix 1 and helix 2 of the homeodomains, and comprises two subgroups of proteins, the KNOTTED1-LIKE homeobox (KNOX) and BEL1-like homeobox (BLH/BELL) [1]. KNOXs are further subdivided into two classes, class 1 (KNOX1) and class 2 (KNOX2), which play antagonistic roles in patterning plant body and organ development through the modulation of multiple hormonal pathways [2].

TALEs are encoded by two small subfamilies in the *Medicago truncatula* genome, which contains 10 genes encoding KNOX TFs, two genes encoding shorter proteins lacking the homeodomain (MtFCL1-2), and 13 *BLH/BELL* genes. Three *KNOX2* genes, belonging to the *KNAT3/4/5-like* subclass (*MtKNOX3, MtKNOX5* and *MtKNOX9*), play a role in symbiotic nodule formation through the indirect activation of the MtEFD/MtRR4 cytokinin-related regulatory module [3]. The involvement of specific TALE TFs in root symbiosis, and in the hormonal networks underlying symbiotic nodule formation, is as yet unclear.

To better characterize the role of the TALE TFs in the regulatory networks underlying rhizobia-induced nodule development, we analyzed transcriptomics data by applying a “targeted” knowledge-based bioinformatics approach developed in our lab. In particular, we focused on the relationship between hormonal networks and *TALE* genes which expression is modulated during nodule formation and behave as “hubs” in the regulatory gene co-expression network (GCN) underlying legume root symbiosis. Possible targets in the hormonal pathways of cytokinins and gibberellins were predicted and validated.

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AURORA KINASE INTERACTS WITH MICROTUBULE-ASSOCIATED PROTEIN AND KINESIN TO REGULATE SYMBIOTIC INFECTION

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Most legumes can form an endosymbiotic association with soil bacteria called rhizobia, which colonize specialized root structures called nodules where they fix nitrogen. This endosymbiosis requires the formation of unique transcellular structures called infection threads that mediate bacterial entry into root cells, but we know very little about how they are formed. Infection thread formation involves a dramatic reorganization of the cytoskeleton and new cell wall deposition. Our results showed that Aurora kinase 1 (AUR1), which has a conserved role across eukaryotes in spindle formation during mitosis, is crucial for symbiotic infection by rhizobia [1]. AUR1 can interact with and phosphorylate microtubule-associated protein (MAP65) and kinesin, which likely regulate microtubule bundling dynamics for orderly reorganization of the cytoskeleton. Using genetic approaches, we found that mutation of these genes resulted in similar infection threads defects in *Medicago truncatula*. Therefore AUR1, MAP65 and kinesin might cooperate to regulate changes in the microtubule-mediated cytoskeleton during nodulation. We further showed MYB3R1, a rhizobia-induced mitotic transcription factor, directly activates the expression of *AUR1* and other cell cycle genes. Our findings indicate that a conserved mitotic module was recruited in legumes for infection thread formation.

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USING PHYLOGENOMICS IN LEGUMES TO UNCOVER NOVEL NODULATION GENES

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To ensure optimal nodulation, a coordinated response is required between the plant and its environment as well as between different organs within the plant. This genetic and molecular regulation involves a complex cascade of local and systemic signalling components to promote or inhibit nodulation [1]. Phylogenomics has emerged as an integral tool in the effort to help uncover potential components acting in these pathways.

Using legume centred gene family phylogenomics, together with iterative gene searches[2] and functional characterisation[3], we have successfully identified CLAVATA3/Endosperm Surrounding Region-related (CLE) peptides in *Medicago truncatula* that are negative regulators of nodule development, acting in the Autoregulation of Nodulation and nitrate regulation of nodulation pathways[3]. This same approach has been used to identify novel peptides of *Glycine max* in the *Root Meristem Growth Factor* (RGF) gene family that regulate nodule maturation and root growth[4] and are analogous to those identified in *M. truncatula*[5]. Here, we will present our findings on the use of phylogenomics with non-legume and legume species to identify potential genes involved in the regulation of nodule development and control.

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FROM LATERAL ROOT TO FUNCTIONAL NODULE: SPATIOTEMPORAL UNDERSTANDING AND ENGINEERING ORGANOGENESIS IN BARLEY

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Since crop yields are often limited by nitrogen availability [1], scientists have been investigating the potential of leveraging biological nitrogen fixation through the legume-rhizobium symbiosis, for crop engineering. Based on our current understanding of the root nodule symbiosis [2], we aim to produce self-fertilizing crops by transferring this symbiotic relationship with nitrogen-fixing rhizobia from legumes to cereals. Establishing a functional symbiotic relationship between rhizobia and plants requires tightly regulated nodule organogenesis. Although our understanding of root nodule symbiosis in legumes has progressed dramatically, acquiring spatiotemporal resolution over the mechanisms involved in lateral root organogenesis in target crops is essential for transferring this trait to non-nodulating species. Our research projects are investigating existing developmental mechanisms in barley lateral root with spatiotemporal perspectives and utilizing this knowledge to conduct developmental-stage-specific and tissue-specific engineering on barley nodule-like structures to accommodate nitrogen-fixing bacteria.

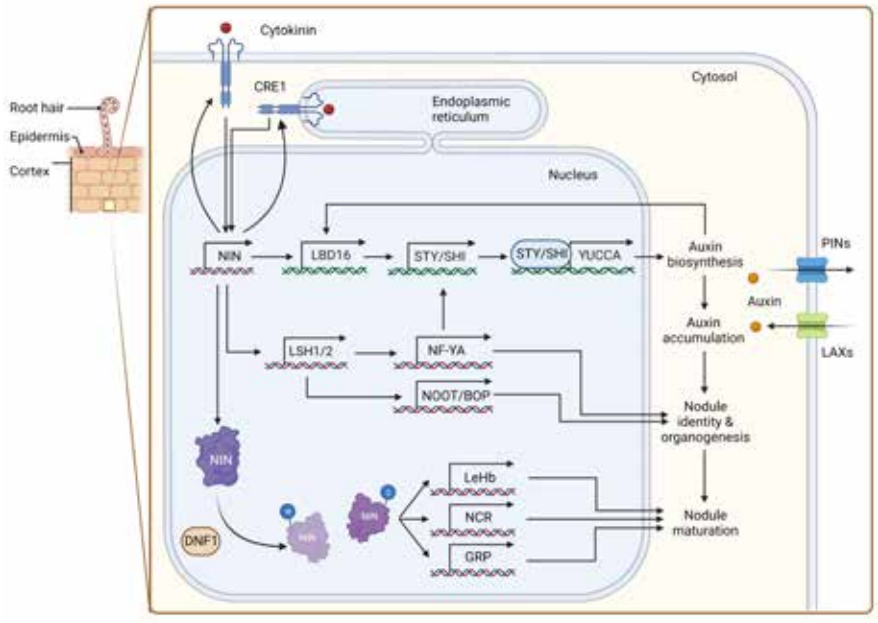

Figure 1. The regulatory network of legume nodule development [2].

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ESSENTIAL ROLE OF NODULE INCEPTION IN SYMBIOTIC NITROGEN FIXATION

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Legumes and actinorhizal plants establish symbiotic relationships with nitrogen-fixing bacteria, resulting in the formation of nodules. Nodules create an ideal environment for nitrogenase to convert atmospheric nitrogen into biological available ammonia. NODULE INCEPTION (NIN) is an indispensable transcription factor for all aspects of nodule symbiosis. Recently, we reported that NIN regulates the transition to nitrogen fixation via proteolytic processing by a signal peptidase complex. Cleavage of NIN results in a carboxyl-terminal fragment containing the DNA binding domain, which activates a series of genes associated with symbiosome development and nitrogen fixation. Thus, proteolytic processing of NIN serves as a critical checkpoint in legume nodulation.

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NITFIX: PHYLOGENOMIC DISCOVERY AND ENGINEERING OF NITROGEN FIXATION

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Our overall goal is to discover the underlying genome novelties that evolved the mutualistic symbiotic relationship between plants and N-fixing bacteria. Identifying the evolutionary origins of root nodule symbiosis (RNS) is critical for understanding its genetic basis. Thus, we reconstructed the evolution of RNS by developing the most extensive *de novo* phylogeny for any lineage and an enhanced nodulation trait database that includes almost 15 thousand species of the N-fixing clade (NFC)[1]. Guided by the refined phylogeny, we aligned the genes and genomes of nodulators, non-nodulators and outgroup species and identified 3,091 conserved noncoding sequences (CNS) among N-fixing species in the NFC[2] , as well as orthogroups associated with the evolution of the nodulation trait. Accessibility of these CNS and genes was assessed with transposase-accessible chromatin sequencing (ATAC-Seq) in Medicago and combined with gene regulatory features to predict the temporal transcriptome (RNA-seq) dynamics of roots treated with rhizobia lipo-chitooligosaccharides (LCO)[3]. This allowed us to identify *cis*-regulatory elements and associated transcription factors that most significantly contribute to the root transcriptome changes triggered by LCOs. While informative, cell type-specific gene expression changes were masked in this analysis of whole tissues. To address this limitation, we have now established a gene expression atlas of the changes that occur in each lineage that originates nodule compartments in Medicago, using single-cell RNA-seq methods[4]. This single-cell atlas allows us to evaluate the role of genes and putative regulatory sequences along cellular lineages, to identify regulators of nodule formation that may be needed to engineer its organogenesis in non-nodulators.

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PRODUCTION OF NODULE-SPECIFIC PEPTIDE MUTANTS AND SCREENING FOR SYMBIOTIC PHENOTYPE

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Most of the legumes form nitrogen-fixing symbiosis with Rhizobium bacteria, in the newly developing symbiotic organ, the root nodule. In the nodule cells, the symbiotic bacteria, the so-called bacteroids, are encapsulated in the peribacteroid membrane originating from the plant plasmamembrane, which serves as a barrier to separate the partners from the each other but also allows exchange of nutrients and peptides between them. In the inverted repeat-lacking clade (IRLC) of legumes a unique gene family with more than 700 members, the nodule-specific cysteine-rich (NCR) peptide family evolved to induce and direct an irreversible, terminal differentiation of the bacteroids, causing genome amplification, inhibition of cell division and resulting in cell elongation, increased membrane permeability and unviable cells outside of the nodule[1]. Another group of nodule-specific transcripts that are restricted to the IRLC legumes are genes encoding glycine-rich proteins (nodGRPs). Their first members in the *Medicago* genus were described in *M. sativa*[2], while the entire set of its genes was collected in the model legume *M. truncatula* where the nodule-specific expression of several GRP genes was shown[3]. Since only a few *NCR* gene mutants are available and revealed a symbiotic phenotype[4,5,6], we aimed to screen for more *NCR* and *GRP* genes for possible symbiotic defects using RNAi approach. We designed and cloned RNAi constructs for several *M. truncatula NCR* and *GRP* genes and produced transgenic plants with them. The offspring of the transgenic plants have been used for testing the plant for their symbiotic phenotypes and these results will be presented.

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EXPLORING THE FUNCTION OF THE *MtnodGRP3C* GENE IN THE DEVELOPMENT OF NITROGEN FIXING NODULES

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Symbiotic nitrogen fixation takes place in the root nodules of legumes, in which Rhizobium bacteria transform into nitrogen-fixing bacteria capable of reducing the atmospheric nitrogen gas to ammonia. In the nodules of the inverted repeat-lacking clade (IRLC) of legumes, hundreds of plant peptides are produced in the symbiotic cells, most of them belonging to the NCR family, which play a role in the terminal differentiation of bacteroids[1]. Another gene family coding for nodule-specific glycine-rich proteins (nodGRPs), has also co-evolved with NCRs in the IRLC plants. The first nodGRPs were described in *M. sativa*[2]. The genome sequence of the model legume *M. truncatula* made possible to identify the entire set of *nodGRP* genes and similarly to the *M. sativa nodGRPs*, the tested members of *MtnodGRP*s also exhibited nodule-specific expression[3].

In this work we focused on *MtnodGRP3C* (*Medtr2g069245*), and investigated its possible role during symbiotic nodule development by using different approaches. The *MtnodGRP3C* gene is located on chromosome 2 and followed by two other members of the *MtnodGRP3* family. *MtnodGRP3C* is highly expressed in nodule zone II where differentiation of bacteroids begins and at lower level in the interzone where the major differentiation of the bacteroids occurs. Silencing of *MtnodGRP3C* in transgenic plants resulted in the formation of small and white nodules. Elongation of bacteroids occured but they were unable for nitrogen fixation and the incomplete bacteroid and symbiotic cell differentiation resulted in early nodule senescence. We also created constructs overexpressing the *MtnodGRP3C* gene from different constitutive promoters. Their possible effect on symbiosis was already tested on transgenic hairy roots while production of stable transgenic plants with these constructs is underway. Microscopic analyses revealed interesting features of these nodules that will be presented as well as possible interacting partners of MtnodGRP3C.

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IDENTIFICATION AND CHARACTERIZATION OF COMMON BEAN (*Phaseolus vulgaris*) NON-NODULATING MUTANTS ALTERED IN RHIZOBIAL INFECTION[1]

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The symbiotic N_2-fixation process in the legume–rhizobia interaction is relevant for sustainable agriculture. The characterization of symbiotic mutants, mainly in model legumes, has been instrumental for the discovery of symbiotic genes, but similar studies in crop legumes are scant. To isolate and characterize common bean (*Phaseolus vulgaris*) symbiotic mutants, an ethylmethanesulphonate-induced mutant population from the BAT 93 genotype was analyzed. Our initial screening of *Rhizobium etli* CE3-inoculated mutant plants revealed different alterations in nodulation. We proceeded with the characterization of three non-nodulating (*nnod*), apparently monogenic/recessive mutants: *nnod*(1895), *nnod*(2353) and *nnod*(2114). Their reduced growth in a symbiotic condition was restored when the nitrate was added. A similar *nnod* phenotype was observed upon inoculation with other efficient rhizobia species. A microscopic analysis revealed a different impairment for each mutant in an early symbiotic step. *nnod*(1895) formed decreased root hair curling but had increased non-effective root hair deformation and no rhizobia infection. *nnod*(2353) produced normal root hair curling and rhizobia entrapment to form infection chambers, but the development of the latter was blocked. *nnod*(2114) formed infection threads that did not elongate and thus did not reach the root cortex level; it occasionally formed non-infected pseudo-nodules. The current research is aimed at mapping the responsible mutated gene for a better understanding of SNF in this critical food crop. Following the whole genome sequence analysis approach, comparing BAT 93 vs each *nnod* mutant sequences, has allowed identifying high and moderate impact sequence variants in six *nnod*(2353) candidate symbiotic genes sequence, that are being further analyzed to identified the causal gene of the mutated phenotype.

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BHLH/HLH HETERODIMER REGULATES NODULE VASCULAR BUNDLE POSITION AT PERIPHERY IN MEDICAGO

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Legumes develop nodules with peripheral vascular bundle that aids in higher nitrogen fixation efficacy compared to non-legumes with central vasculature. The evolutionary shift in vascular bundle from centre to the periphery of nodule has been less explored. In this study, we show that *bHLH1/NVD1* (renamed *Nodule Vascular Bundle Development 1*) gene play role in peripheral positioning of vascular bundle in Medicago nodules. *nvd1* nodules forms partially central positioning of vasculature, distorted nodule shape, and increased nodule length, leading to decreased nitrogen fixation efficiency and fresh weight of plants. Further, we found that NVD1 target and activates HLH transcription factor named *NVD2*, which heterodimerises with NVD1, thus regulating NVD1 activity by forming NVD1-NVD2 non-functional units. The shape and length of *nvd2* nodules remain unaffected but possess central vasculature defect similar to *nvd1* with a more pronounced defect towards the nodule meristematic zone. *NVD2* express in nodule vasculature, cortical layers 3,4,5, pericycle, and endodermal cell layer in developing nodule primordium and specifically in nodule vasculature and nodule vascular meristem in mature nodule. NVD2-GFP fusion protein localizes specifically to the nucleus of nodule vascular endodermis. Both *NVD1* and *NVD2* are auxin-inducible genes. We identified that Auxin Responsive Factor 5 (ARF5) directly bind and activate both genes in a transactivation assay in heterologous tobacco leaf cells. However, for auxin-induced *NVD2* expression in Medicago NVD1 is necessary. Transcriptomic study of *nvd1* and *nvd2* nodules emphasize their role in nodule meristem formation/ maintenance. We demonstrated nodule development is tilted towards the root development in *nvd1* and *nvd2* nodules. Finally, the auxin and cytokinin signaling is disrupted in *nvd1* and *nvd2* nodules. We developed a hypothetical model that show NVD1 and NVD2 is activated downstream of Nod Factor (Nodulation Factor or NF) signalling pathway. The detailed role of NVD1 and NVD2 will be presented.

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THE POTENTIAL OF FLEMISH RHIZOBIA AS SOYBEAN INOCULANTS

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More than 90% of the soybean used in the European Union is imported[1], endangering vulnerable ecosystems, especially in South America[1]. Increased local production would make soybean consumption more sustainable. Already, early-maturing soybean varieties have been cultivated and screened to cope with the colder climate[1]. However, even though many commercial inoculants are available as symbiotic partners for soy, they are not adapted to the North-West European conditions, resulting in suboptimal yields. Indeed, strains adapted to the local soil and environment show high potential as inoculants[2].

As part of the citizen science project 'Soybean in 1000 Gardens', a large-scale isolation campaign was set up in Flanders to find indigenous rhizobial species. Bacteria were isolated from soybean root nodules resulting in a collection currently comprising over 600 unique strains. Eleven *Bradyrhizobium* strains have been sequenced via Illumina and Oxford Nanopore Technologies. Even though all *Bradyrhizobium* strains were isolated from root nodules, only four out of eleven strains seem to contain the *nodABCD, nifA*, *nifHDK,* and *fixABC* genes for nodulation and nitrogen fixation. Thus, the other seven strains might be free-living *Bradyrhizobium* strains, which contradicts the fact that they were isolated from nodules. To check the nodulation capacity of these strains, *in planta* experiments are being performed. The formation of the nodule structures will be microscopically compared, which allows the coupling of genomic with phenotypic data. This will help our understanding of the interaction between the bacteria and the plant and how it can be further optimized.

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BIOLOGICAL NITROGEN FIXATION BY SOYBEAN (*GLYCINE MAX* [L.] MERR.), A NOVEL, HIGH PROTEIN CROP IN SCOTLAND, REQUIRES INOCULATION WITH NON-NATIVE BRADYRHIZOBIA

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It is currently not recommended to grow soybean further than 54° North, but climate change and the development of new high latitude-adapted varieties raises the possibility that it could be introduced into Scotland as a novel high protein crop deriving most of its nitrogen (N) requirements through biological N fixation (BNF). This was evaluated via field trials in 2017 and 2018 near Dundee (56.48°N). As there are no native soybean-nodulating bacteria (SNB) in UK soils, soybean requires inoculation to exploit its BNF potential. In 2017, three commercial inoculants containing elite *Bradyrhizobium* strains significantly increased plant biomass in plot trials with a soybean 000 maturity group variety (ES Comandor). Rhizobia were isolated from the nodules and identified as the original inoculant species, *B. diazoefficiens* and *B. japonicum*. One inoculant (Rizoliq Top) was used for larger-scale trials in 2018; inoculation doubled the grain yield to 1 t ha^{-1} compared to the uninoculated crop. The inoculated soybean obtained most of its N through BNF in both years regardless of plant genotype i.e. >73%Ndfa, with BNF contributions to aerial biomass exceeding 250 kg N ha^{-1} yr^{-1} in 2017 and that to grain 50 kg N ha^{-1} yr^{-1} in 2018. These data suggest that N-fixing soybean could be grown in Scotland without mineral N-fertiliser. The potential for survival of the *Bradyrhizobium* inoculant strains in soils was also demonstrated through the detection of the inoculant strain *B. diazoefficiens* SEMIA 5080 at relatively high populations (10^4 g^{-1} dry soil) using a qRT-PCR method with SNB-specific *nodZ* primers.

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UNRAVELLING CELL WALL MODIFICATION MACHINERY ASSOCIATED WITH THE INTERCELLULAR INFECTION IN PEANUT

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Nitrogen-fixing endosymbiotic interaction via de novo nodule organogenesis evolved ~110 million years ago in the common ancestors of the nitrogen fixation clade (NFC). Phylogenomic studies reveal the evolution and acquisition of nitrogen-fixing traits in nodulating lineages as well as the loss of several genes in the non-nodulating relatives in parallel, highlighting an evolutionary pressure is operational to stabilize this trait. While the majority of the legumes show an intracellular mode of rhizobial infection by infection thread (IT), about 25% of the legumes exhibit intercellular infection, a primitive mode of invasion. Since intercellular invasion is less sophisticated than intracellular mode, it can be a useful tool to engineer nitrogen fixation in crops. To date, the molecular players determining intercellular infection remain elusive. Peanut diverged from the model legumes ~ 50-55 million years ago and appears to be present at an evolutionary crossroad, exhibiting primitive intercellular infection and endoreduplication of the rhizobial genome which is considered an advanced feature[1]. Previously we demonstrated that *Nodule Inception* (*NIN*), the master regulator of nodulation, is not involved in epidermal crack entry and is only expressed in the cortex after infection. Additionally, orthologs of several direct targets of NIN, are either absent in peanut genome or do not induce during nodule development. We have shown that a Glycosyl Hydrolase (GH) family member regulates intercellular crack entry, instead of *Nodulation Pectate Lyase* (*NPL*) implicated in IT-mediated infection[2]. This highlights the recruitment of different natures of cell wall modification during the intercellular mode of infection. How different players orchestrate the remodeling of the cell wall during crack entry will be discussed.

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GENOMIC ANALYSIS OF A *SINORHIZOBIUM* STRAIN ISOLATED FROM THE TUNISIAN DESERT

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The N_2-fixation by rhizobia in symbiosis with legumes is critical to global nitrogen cycling and sustainable agriculture[1] and for survival and prevalence of endemic spontaneous legume's in Arid and Saharan regions of Tunisia. The strain *Sinorhizobium meliloti* IRAMC:0087 was originally isolated from root nodules of a Saharan shrub *Calobota saharae* growing in the Saharan regions of Southern Tunisia. IRAMC:0087 can nodulate *Acacia tortilis* var. *raddiana*, a plant-tree adapted to extreme climatic conditions, and endophytically colonize *Trifolium subterraneum* roots. Phenotypic characterization of this strain has revealed tolerance to high salinity levels, drought, and high temperatures. To further investigate the molecular basis of this strain´s behavior, we sequenced its complete genome. The genome comprises 5 replicons, a chromosome (3,650,495 bp), the p*SymA* and p*SymB* (1,247,198 and 1,674,059 bp) replicons, and two additional plasmids (597,953 and 197,378 bp) with a GC content of 61.94%. In total, 6,558 protein-encoding sequences, 56 tRNAs and 9 rRNAs along with an intact prophage of 53.3 kb with similarity to *Sinorhizobium* phage phiLM21 were identified. The genome encodes gene clusters supporting rhizosphere processes, secondary bioactive metabolites, plant growth-promoting activities and symbiosis. Interestingly, one of the additional plasmids encodes several genes and gene clusters related to stress tolerance, namely trehalose and osmoprotectant biosynthesis, which may contribute to the adaptation of this strain to severe conditions. IRAMC:0087 exhibits an endophytic and symbiotic behavior with hosts adapted to extreme climatic conditions. Comparative genomic analyses with other rhizobial strains have the potential to reveal novel factors mediating symbiosis under those conditions.

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CROSS-COMPATIBILITY OF RHIZOBIA TO MAXIMISE NITROGEN FIXATION IN THE NEW ANNUAL PASTURE LEGUME *SCORPIURUS MURICATUS*

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Agricultural productivity in medium-to-low rainfall areas of southern Australia is constrained by a lack of annual pasture legumes suitable to this region. *Scorpiurus muricatus* is an annual pasture legume classified in tribe *Loteae* from the Mediterranean basin, with potential to fill this niche. All agricultural legumes in Australia are exotic and do not form effective symbioses with indigenous rhizobia, so a highly effective inoculant strain for *S. muricatus* needs to be identified. There is very little information on the diversity, effectiveness, and host-range of *S. muricatus* microsymbionts. Additionally, understanding the promiscuity of *S. muricatus* is essential as inoculants for other legumes and pre-existing soil bacteria have the potential to reduce *S. muricatus* yield if they fix N_2 suboptimally on this host.

A total of 39 authenticated strains were isolated from soils from Australia (5), Croatia (2), Israel (11), Morocco (4) and Sardinia (17). Core genome analysis has identified several potential novel *Mesorhizobium* species. Symbiotic effectiveness testing of 36 strains inoculated on *S. muricatus* showed 35 strains produced equivalent shoot dry weight (SDW) to the reference strain WSM1386 and one strain produced significantly less. The most effective strain WSM4842 produced 149% of the SDW of WSM1386, this result warrants further investigation into WSM4842 as a potential inoculant.

S. muricatus promiscuity was investigated by inoculating plants with Australian commercial *Mesorhizobium* and *Bradyrhizobium* inoculants. *Mesorhizobium* inoculants for *Lotus corniculatus* and *Lotus ornithopodioides* nodulated and fixed N_2 on *S. muricatus* while *Bradyrhizobium* inoculants for *Lotus pedunculatus*, *Lupinus* spp. and *Ornithopus* spp. nodulated but did not fix N_2. Furthermore, *S. muricatus* is nodulated by resident *Bradyrhizobium* with initial testing suggesting they fix N_2 ineffectively. Future experiments will quantify effectiveness and saprophytic competence of effective *Mesorhizobium* strains in field conditions and in sites with a history of cultivation with other *Mesorhizobium*-nodulating legumes.

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GENETIC ANALYSIS OF PLANT ROOT ENDOSYMBIOSES IN *DRYAS* (ROSACEAE)

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To overcome nutrient limitations, plants engage in two main types of root endosymbioses with beneficial microbes. Arbuscular mycorrhiza (AM) is an ancient symbiosis formed by about 70-90 % of land plants and phosphate-acquiring fungi of the phylum Glomeromycota. By contrast, the root nodule symbiosis (RNS) with nitrogen-fixing bacteria is phylogenetically restricted to a single clade encompassing four angiosperm orders – Fabales, Fagales, Cucurbitales and Rosales – within which the distribution of RNS-forming species is scattered[1, 2]. The order Rosales encompasses the economically important family Rosaceae (~3000 species) which comprises four RNS-forming genera that all belong to the Dryadoideae subfamily[3, 4]. From an evolutionary perspective, the genus *Dryas* is particularly interesting because it contains the closely related RNS and non-RNS-forming species *Dryas drummondii* and *Dryas octopetala*, respectively[5, 6, 7]. This genetic polymorphism within the genus *Dryas* holds the potential to discover new genetic element(s) required for the establishment plant root endosymbioses[8]. To identify the causative mutation(s) responsible for the polymorphic symbiosis trait within the *Dryas* genus, we made used of a hybrid species emanating from the cross between *D. drummondii* and *D. octopetala*. We *de novo* sequenced the genomes of *D. drummondii*, *D. octopelata* and the *D. drummondii* × *D. octopelata* hybrid using the PacBio sequencing technology and inspected the ability of the putative F2 population to establish plant root endosymbioses.

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THE ROLE OF ICESYM IN SYMBIOTIC PERFORMANCE OF CHICKPEA MESORHIZOBIA

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Horizontal gene transfer of symbiotic integrative conjugative elements (ICESym) contributes to diversity of chickpea mesorhizobia. ICESyms carry nodulation and nitrogen fixing genes which are transferred to non-nodulating mesorhizobia in soil affording them the ability to nodulate chickpea hosts. Australian soils provide a unique environment for this diversity to develop and evolve as the native rhizobia are unable to nodulate chickpea [1]. In Australia, *Mesorhizobium ciceri* strain CC1192 has been the only commercial inoculant strain for chickpea since its introduction in the 1970s, therefore ICE*Mc*Sym^{1192} is likely to be the only ICESym found in chickpea nodule isolates [2]. Studies thus far have demonstrated that symbiotic performance of novel nodulating isolates containing ICE*Mc*Sym^{1192} can vary in plant assays but is not significantly different to CC1192 in field experiments [2;3;4]. Due to the lack of genetically diverse ICESyms found in Australian soils, the resulting symbiotic performance may be limited [4]. The aim of this research is to determine the diversity of ICESyms in mesorhizobia isolated from Australian field grown chickpea and investigate the role of diverse ICESyms in the symbiotic performance of chickpea mesorhizobia. The Pulsford laboratory at the University of Sydney has a large collection of chickpea mesorhizobia, including nodule isolates from Australian soils, nodulating mesorhizobia with genetically diverse ICESyms and an ICESym-cured variant of CC1192. Preliminary results indicate that ICESyms from Australian nodule isolates are derived from CC1192 and that introduction of three different ICESyms, two monopartite and one tripartite, results in variable symbiotic performance. Introduction of diverse ICESyms to Australian soils may increase symbiotic performance of chickpea mesorhizobia in Australian soils.

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COMPLETE GENOME SEQUENCING AND PHYLOGENETIC ANALYSIS OF THE AUSTRALIAN COMMERCIAL RHIZOBIAL INOCULANTS

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Rhizobia compatible with crop and pasture legumes aren't naturally present in Australian soils, which has led to the introduction of many highly effective nitrogen-fixing bacteria from other parts of the world as commercial inoculant strains. Rhizobial symbiosis genes (*nod*/*nif*/*fix*) are encoded from mobile genetic elements, such as plasmids or integrative and conjugative elements (ICEs). As a result they are highly mobile and able to transfer between strains [1].

Recently, it has become increasingly clear that the genetic diversity of strains nodulating legumes far exceeds the diversity of strains introduced as inoculants [2-5]. This could partly be explained by horizontal transfer of symbiosis genes between inoculants and pre-existing soil bacteria, creating novel hybrid rhizobia [6-8]. It could also be due to the accumulation of mutations giving rise to newly evolved rhizobia.

Given the genetic instability of the symbiosis genes, a blueprint for inoculant genomes is important to maintain the integrity of this crucial national resource. However, we lack the genomic data on the commercial legume inoculants needed to track changes in genome structure and content as well as the technology to easily identify strains within nodules. In this project, we describe the complete genome sequencing of the Australian commercial rhizobial inoculants, with special attention paid to the symbiosis genes, and examine their relationship to each other, as well as to the broader set of publicly available genome sequences. This critical baseline data can be used to address fundamental issues surrounding inoculant usage including the identity of the bacterium within a nodule and how the inoculants change over time.

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ISOTOPE RATIO ANALYSES TO INVESTIGATE NITROGEN FIXATION IN SYMBIOTIC LUCINID CLAMS

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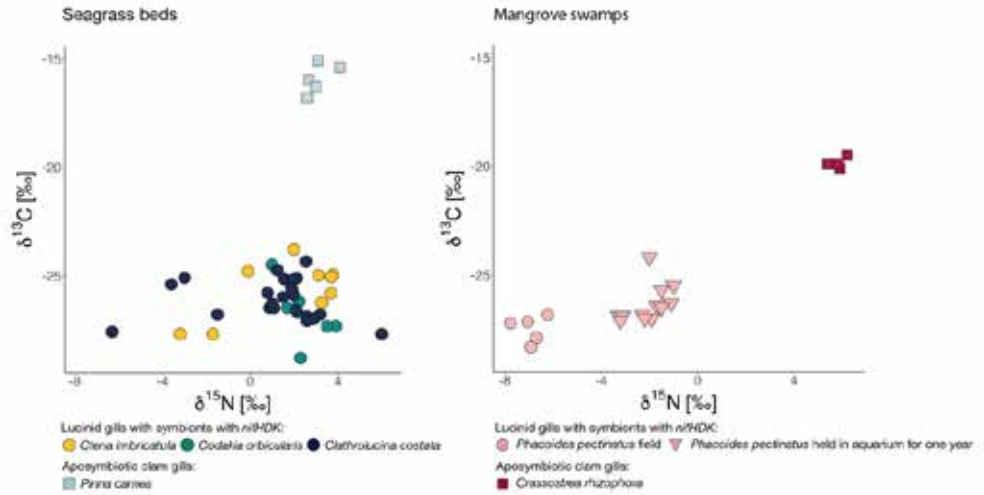
Bivalves of the family Lucinidae harbour sulfur-oxidizing Gammaproteobacteria in their gills, which fix inorganic carbon and provide organic carbon to their host[1]. In oligotrophic environments, such as coastal habitats on the Caribbean side of the Isthmus of Panamá, these symbionts possess genes affiliated with nitrogen fixation[2]. It is hypothesized that the uptake of these horizontally transmitted symbionts helped their hosts to adapt to the nitrogen limited environment. However, it has not been shown that the symbionts fix nitrogen in association with the clams and, if so, pass it on to their host. Investigating isotopic ratios of dried lucinid clam gills and aposymbiotic control filter feeders in the same habitat indicate carbon fixation. Measuring natural d^{15}N values makes us raise the question whether nitrogen fixation is conducted during the symbiosis or if it might be a trait of the free-living stage of the bacteria. Under varying laboratory set-ups, we were not able to show nitrogen fixation in different lucinid species. In addition, analysed isotopic ratios of lucinid visceral masses and shells do not indicate a passing on of potentially fixed nitrogen from the primary producer, the bacteria in the gills, to other clam tissues.



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COMPLETE GENOMES UNVEIL HIDDEN TAXONOMIC AND FUNCTIONAL FEATURES WITHIN THE GENUS *SINORHIZOBIUM*

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The genus *Sinorhizobium* comprises rhizobial species commonly known for their ability to fix nitrogen in symbiosis with legumes. Amongst the species of this genus*, S. meliloti* has extreme importance due to its status as a model rhizobium for studying the processes occurring during the symbiotic interactions between rhizobia and legumes [1]. The genus *Sinorhizobium* was recently subjected to several taxonomic rearrangements [2]. To support taxonomic studies of this genus and of rhizobia more broadly, we report complete genome sequences and annotations for the species type strains *S. garamanticum, S. numidicum* and *S. kummerowiae*, which were not previously available. Comparative analyses of these genomes with those available in public databases lead to the reclassification of the type strain *S. kummerowiae* CCBAU 71714^T into the species *S. meliloti*. No reclassification is needed regarding the type strains of the species *S. garamanticum* and *S. numidicum.* Moreover, we sequenced the complete genome of strain MABNR56, a rhizosphere bacterium isolated from the root rhizosphere of *Brassica napus* (rapeseed). This strain was identified as *S. meliloti* by phylogenetic analyses using 16S rRNA gene sequences or 92 housekeeping genes. Functional annotation of its complete genome revealed that this strain possesses some interesting capacities and potential agro-ecological importance for its hosts other than biological nitrogen fixation. Overall, our latest results showed that further reclassification of species, as well as new functionalities, might remain to be uncovered within the genus *Sinorhizobium.*

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DIFFERENT SPECIES OF *BRADYRHIZOBIUM* FROM SYMBIOVARS GENISTEARUM AND RETAMAE NODULATE THE ENDEMIC *RETAMA DASYCARPA* IN THE HIGH ATLAS MOUNTAINS

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The genus *Retama*, (Genisteae, Papilionidae, Fabaceae) includes four validly described shrubby perennial species adapted to arid environments (*R. monosperma* (L.) Boiss., *R. raetam* (Forssk.) Webb, *R. sphaerocarpa* (L.) Boiss. and *R. dasycarpa* (Cosson.)) [1]. *Retama dasycarpa* is endemic to Morocco and its geographical distribution is limited to the High Atlas Mountains; whereas the other 3 species are found in North African countries, Southern Europe, the Canary Islands and eastern Asia [1].

Several studies reported that Retama spp. are most commonly nodulated by species of the genus *Bradyrhizobium* [1]. However, Rejili et al. reported that R. raetam is nodulated by *Ensifer meliloti* in arid environments in Tunisia [2]; while *Ensifer aridi* nodulates *R. monosperma* in eastern Morocco [3]. More recently, Lamrabet et al [1] reported the isolation of members of the genus *Microvirga* from nodules of Retama spp. in the Maamora forest, Morocco. However, to date, no studies have been conducted on the endosymbionts of the endemic *R. dasycarpa*. Therefore, in this paper, our main objective was to characterize for the first time the diversity of nodulating bacteria associated with this endemic plant in different locations of the High Atlas Mountains.

We analyzed the molecular, phenotypic and symbiotic diversity of microsymbionts of *R. dasycarpa*. Phylogenetic analysis of the 16S rRNA gene revealed that the isolates clustered in the genus *Bradyrhizobium*, while MLSA of *recA*, *gyrB*, *glnII* and *atpD* grouped the 12 selected strains into four groups close to *B. lupini*, *B. frederickii, B. valentinum* and *B. retamae*. Phylogenies of the symbiotic genes *nodC*, *nodA* and *nifH* revealed that the strains are members of the genistearum and retamae symbiovars. The isolates have a wide host range, being able to nodulate different Genisteae, but not *Phaseolus vulgaris*, or *Glycine max*. This work is the first description of the microsymbionts associated with *R. dasycarpa*.

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INFERRING LOSS AND TRANSFER OF NITROGEN FIXATION GENES IN THE PHYLOGENY OF BACTERIAL LUCINID SYMBIONTS

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Bivalves of the family Luncinidae (lucinids) harbor chemosynthetic sulfide-oxidizing Gammaproteobacteria in their gills, which synthesize organic carbon that serves as nutrition for their host [1,2]. Lucinids of the genera *Ctena* and *Codakia* are distributed across the Isthmus of Panamá and their sister species can be found respectively on each side of the Isthmus (Atlantic and Pacific)[3,4]. When the Isthmus closed ~2.8 MYA, these lucinids and their bacterial symbionts had to adapt independently to slowly diverging environmental conditions (nutrient availability, temperature, salinity, etc.)[5]. It has been hypothesized that the bacterial symbionts play a crucial role in their hosts adaptation[6], but in the case of lucinids across the Isthmus, this role is still unknown. Through the analysis of the gene content of the symbionts' metagenome assembled genomes (MAGs), we discovered that only symbionts from the Atlantic side of the Isthmus have the genomic potential for nitrogen fixation, even though they are very closely related to the symbionts found on the Pacific side (some could even be considered the same species). By reconciliating the symbiont species tree and the nitrogenase gene tree, we revealed that this pattern is a product of several events of loss and regain – through horizontal gene transfer - of nitrogen fixation genes and that these events seem to be linked to nutrient availability in the environment. These observations support the idea that bacterial symbionts, which have access to a wider genetic pool for genetic exchange, can lead to a fast adaptive response [7] and allows the symbiosis to exploit new resources and cope with new habitats [6,8,9].

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THE PAN-EPIGENOME OF THE SYMBIOTIC NITROGEN FIXING BACTERIUM *SINORHIZOBIUM MELILOTI* UNRAVELS UNEXPECTED COMPLEXITY OF DNA-METHYLATION PROFILES

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In prokaryotes, DNA methylation has been found to be involved in several phenomena, from DNA repair to cell cycle progression, gene transfer and regulation. Here, we investigated the presence of strain-by-strain variability in genome-wide methylation pattern by performing an epigenomic analysis on 21 strains of the facultative plant symbiotic nitrogen-fixing alphaproteobacterium *Sinorhizobium meliloti*. Strains of these species are characterized by a divided (multipartite) genome structure, including a chromosome, a chromid and a megaplasmid, and shows extensive genomic and phenotypic variation, providing good models to test evolutionary hypotheses on the relationships among epigenomic signatures, genome structure evolution and phenotype changes.

Results from SMRT sequencing and analysis with MeStudio software[1] showed the presence of a wide pan-epigenome with 16 DNA methylated motifs, including both 4mC and 6mA palindromic and nonpalindromic motifs. Four motifs only were shared by all strains, while most of the motifs had occurrence in a few *S. meliloti* strains. When inspecting differences among replicons of *S. meliloti*, lower DNA methylation was detected for, possibly recently acquired, accessory plasmids. A differential occurrence of DNA methylation between coding and noncoding regions was also detected for some motifs.

In conclusion, in this work we showed that in *S. meliloti* a large pan-epigenome exists. Further work would investigate the role of such epigenomic differences over phenotypic variation and host plant interaction.

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PATHOGENESIS-RELATED 1 PROTEINS INVOLVED IN LIPID EXCHANGES DURING BACTEROID DIFFERENTIATION AND NITROGEN FIXATION IN PEANUT

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In nitrogen-fixing symbiotic interactions, host plants house their cognate microbes in specialized root-borne organs called root nodules. During the process, the microbial symbionts get enclosed by the host and undergo a range of plant-driven alterations to fix nitrogen. Peanut (*Arachis hypogaea*) exhibits a number of non-canonical nodulation features like, “crack entry” of rhizobia and aeschynomenoid-type nodule development that lacks uninfected cells in the central infection zone. We observed the induction of several genes encoding CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 proteins) superfamily members in peanut nodule developmental transcriptome [1]. We named them Nodule Induced CAPs (NICs). Phylogenetic analyses revealed that majority of the NICs are clustered in a distinct clade suggesting nodule-specific functional divergence of these proteins in peanut. The promoter-reporter assay showed their nodule-specific activity and distinct expression patterns across developmental stages. Proteome analysis of symbiosomes, isolated from nitrogen-fixing mature peanut nodules, confirms that these CAP proteins are secretory in nature and are delivered to the symbiosomes. GFP labelled recombinant proteins and immunolocalization with anti-NIC antibodies revealed that these proteins localize to the symbiosome membranes and are enriched in specific nanodomains on the membranes. We showed that NICs bind to several anionic lipids on membrane lipid strips, among which prokaryotic-specific Cardiolipin (CL) is of particular interest. Knockdown of multiple *CAP* genes in peanut roots resulted in a range of defective structures and fully grown nodules where infection zones contained undifferentiated rhizobia. The observations suggest that NICs are essential for bacteroid differentiation and nodule development. We detected the upregulation of many fatty acid and membrane lipid biosynthetic genes in the nodule transcriptome. We are currently investigating the role(s) of these NIC proteins in the context of bacteroid differentiation and multiplication.

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NODULATION IN THREATENED ENDEMIC MONOTYPIC GENUS INDOPIPTADENIA OUDHENSIS (BRANDIS) BRENAN (LEGUMINOSAE) AND MOLECULAR CHARACTERIZATION OF ITS RHIZOBIA FROM INDIA

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Indopiptadenia oudhensis (Brandis) Brenan is a threatened endemic monotypic genus belonging to mimosoid clade (Caesalpinioideae-Leguminosae). It is a tree species natively distributed in tropical moist deciduous forests of Western and Central Himalayan region; and reported from lower altitudes at the border zone between India and Nepal [1]. This study is the first report on nodulation in *Indopiptadenia* and molecular characterization of its root-nodule microsymbionts. The germinated seeds were transferred in *Indopiptadenia*-rhizospheric soils collected from NBRI, Lucknow, Uttar Pradesh and soils of Botanical Garden JNVU, Jodhpur, Rajasthan. Indeterminate, branched and internally pink root-nodules were observed only in plants grown in NBRI soils whereas they failed to nodulate in alkaline soils of Jodhpur. Our aim was to analyse the genetic diversity of N-fixing microsymbionts associated with *Indopiptadenia* in India and identify them. Thirty seven fast-growing root-nodule bacterial strains were isolated and based on RAPD-fingerprinting patterns using RPO1 primer they clustered into six genotypes. Selective strains for which *nodA* gene could be amplified were identified using *recA* gene sequence as species of *Ensifer* (*Sinorhizobium*). Phylogenetically the strains formed novel clades within *Ensifer* spp. and could be potentially new species. *Indopiptadenia-Ensifer* strains are also phylogenetically distinct from previously reported Indian *Ensifer* strains from various native and invasive mimosoid legumes [2]. Our result indicates that restricted distribution of *Indopiptadenia* in India may be influenced by climatic and edaphic factors as well as availability of its compatible N-fixing microsymbionts which are specific and might have co-evolved with host species which is also an important connecting link in terms of legume systematics.

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DIRECT INVESTIGATION OF A MARINE BACTERIUM ENCODING GROUP VI NITROGENASE

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Nitrogen (N_2) fixation is the largest source of new nitrogen (N) to the global ocean and promotes the sequestration of carbon dioxide from the atmosphere to deeper waters. Phylogenetic analysis split the structural nitrogenase subunits, NifHDK, into a number of distinct clusters with all enzymes from the typical oceanic N_2-fixing microorganisms belonging to cluster I. Recently, a novel cluster (VI) with yet unknown function was discovered within members of the Elusimicrobia from groundwater environments[1]. Here we report the recovery of several cluster VI nitrogenase genes, including one contained in a metagenome assembled genome (MAG), from metagenomes from the South Pacific gyre and the Western Tropical North Atlantic. Phylogenetic analysis suggest that the MAG represents a novel species within the phylum Myxococcota and we tentatively named it *Ca.* Neptunococcus apricus. Using metagenomics plus stable isotope incubations combined with fluorescence in situ hybridization and single-cell analyses (nanoSIMS), we provide the first detailed description, including direct microscopic observations, of a marine group VI nitrogenase harbouring bacterium. While our nanoSIMS results show that *Ca.* Neptunococcus apricus was not fixing N_2 during our experiments, analysis of the genome reveal an otherwise versatile heterotrophic metabolism including the capacity to synthesize cobalamine (Vit. B_{12}), which may be important in interactions with other microorganisms. We further show that *Ca.* Neptunococcus apricus plus the orphan group VI nitrogenase genes are widespread and abundant across the world's oceans, pointing at a possible important role in oceanic microbial communities. Collectively, our results call for further investigations into the functioning of marine group VI nitrogenases and the carrying microorganisms.

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INTERKINGDOM CROSS-TALK BETWEEN SYMBIOTIC NITROGEN FIXING RHIZOBIA AND RHIZOSPHERIC FUNGI: SYNERGISM, ANTAGONISM OR NEUTRALISM IN PLANT GROWTH PROMOTION?

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Aim

In the reconstruction of nitrogen-fixing synthetic communities, a key point is to analyse the presence of biotic relationships between symbiotic nitrogen-fixing rhizobia and the other members of the plant microbiome[1]. Here, as proof-of-concept, we aim at exploring the existence of interactions between rhizobia strains and *Trichoderma* sp. strains, hence synergistic roles of rhizospheric fungi in the symbiotic nitrogen fixation and host plant growth.

Methods

We selected 4 *Sinorhizobium meliloti* strains and 4 *Trichoderma* species. In an experimental scheme of 4 x 4 interactions, we investigated the fungal growth inhibition by dual cultures, the rhizobia growth inhibition and the transcriptomic response elicited by fungal spent media, as well as spent media effects on rhizobia PGP abilities and the effects of the different combinations on the host legume *Medicago sativa*.

Results

Fungal spent media had large and specific x strain specific effect on rhizobia, indicating a general rhizobia genotype x fungal genotype interaction. In particular, a high number of genes was shown to be differentially expressed in rhizobia strains, as well as changes in exopolysaccharide, auxin production and in plant symbiotic phenotypes were identified.

Conclusion

Our results provide a first insight into symbiotic nitrogen-fixing rhizobia and rhizospheric fungi interactions. Given the importance of ensuring more sustainable crop production systems, the dissection of such interactions could contribute the knowledge for a rational use of rhizobia as bioinoculants and development of synthetic communities.

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THE DIVERSITY OF SPECIES WITHIN THE *NITROSPIRILLUM* GENUS

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The *Nitrospirillum* genus was created by dividing *Azospirillum* and reclassifying *A. amazonense* to this new genus[1]. The strains that compose *N. amazonense* were all isolated from Brazil[2], and only recently was the species *N. iridis* described from a strain isolated in China[3]. The main characteristic of the strains from this genus is their ability to fix nitrogen freely, colonize plant roots, and promote plant growth. One strain of the genus (CBAmC, BR 11145) has been used commercially as an inoculant for sugarcane since 2019, following over a decade of laboratory and field studies. Since the early 1980s, hundreds of strains affiliated with *A. amazonense* have been isolated and deposited in the Johanna Döbereiner Biological Resources Center (https://www.embrapa.br/agrobiologia/crb-jd). These strains come from different biomes and hosts in Brazil, with most being isolated from Poaceae. A study based on conserved genes, such as 16S rRNA and recA, genes related to biological nitrogen fixation, genome sequencing analysis, and phenotypic characteristics have indicated that there are more than two hundred *Nitrospirillum* strains in our culture collection, several of which compose phylogenetic groups not yet taxonomically positioned, likely representing new species with unknown biotechnological potential. For example, it was observed that the strain CBAmC needs to be reclassified in other species out of *N. amazonense*, as it is known currently. The detailed characterization of strains within the *Nitrospirillum* genus contributes to a better understanding of bacterial diversity and opens possibilities for studies in the field of biotechnology.

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POSTER
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THE EFFECT OF EXOGENOUS HYPOXIA AND WATERLOGGING ON ROOT NODULE DEVELOPMENT IN LEGUMES

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Legumes are able to establish a symbiotic relationship with rhizobia, a group of bacteria able to convert atmospheric dinitrogen (N_2) into ammonium through the nitrogenase enzyme. In this symbiotic process, rhizobia are localized within specialized root organs called nodules. These organs are physiologically characterized by a microoxic environment that protects nitrogenase from being irreversibly inactivated by oxygen. At the same time, oxygen is required to maintain rhizobia and plant cells metabolism[1]. Hypoxia signalling in plants and animals is now widely characterized[2,3], but only few studies have investigated the activation of this signalling pathway in an intrinsically hypoxic environments such as the nodule, its effect on the symbiotic signalling and in nodule development and function. Furthermore, little information is available about how nodulation and nitrogen fixation are affected by external low oxygen conditions (i.e. flooded soils). To address this understudied aspect of plant physiology, we carried out a phenotypic survey of the effect of exogenous hypoxia and waterlogging on nodule development in legumes that produce either determinate or indeterminate nodules. We observed that when applied at early stages exogenous hypoxia and waterlogging prevent bacterial infection and nodulation, whereas if applied at a later stage, they arrest nodule development. Both effects can be reversed by reoxygenation. We are currently studying the molecular determinants of this phenomenon, by looking at plant and bacterial mechanisms for oxygen sensing.
Understanding these regulatory mechanisms will allow, in the future, the deployment of breeding strategies for improved nitrogen fixation in case of soil hypoxia. It will also support the engineering of nitrogen fixing symbiosis in crop species which do not establish such an interaction with rhizobia.

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CEREAL-ASSOCIATED BACTERIAL ENDOPHYTES PROMOTE NITROGEN FIXATION IN RHIZOBIUM-LEGUME SYMBIOSES

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Biological nitrogen fixation (BNF) by rhizobia during the legume-Rhizobium symbiosis plays pivotal role in improving agricultural productivity and is therefore of great economic interest[1]. Numerous literature data indicate that other beneficial soil bacteria can act synergistically with rhizobia to improve nodulation and growth of several legumes including alfalfa[2]. These bacteria can enhance plant growth by a variety of direct and indirect mechanisms such as nitrogen fixation, phosphate solubilization, production of siderophores and phytohormones, expression of 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) and/or the biocontrol of plant pathogens.

In the present study, we used non-nodulating endophytic bacteria, which were isolated from both Asian (*O. sativa* L.)[3] and African (*O. glaberrima* Steud)[4] rice, to evaluate their synergistic activity. To investigate the role of different endophytes on the nitrogen-fixing ability of legumes, alfalfa plants were inoculated only with rhizobium and co-inoculated with rhizobium and the rice-associated endophytes, and the activity of nitrogenase enzyme measured. This study was initially carried out under greenhouse conditions, then in nutrient deficient soil, and finally under field conditions. We verified that the co-inoculation resulted in enhanced nodulation and nitrogen-fixation efficiency, which led to an increase in biomass production. We hypothesized that these endophytic bacteria could influence the growth of the host plant more efficiently than the inoculation with rhizobium alone, even under field conditions.

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EXPLORATION OF GENETIC DETERMINANTS OF SALT TOLERANCE IN *SINORHIZOBIUM MELILOTI* STRAINS

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Salinity is one of the largest constraints in agriculture for plant growth in stricken regions [1] able to affect the rhizosphere bacterial community [2], and inhibits nitrogen metabolism (e.g. nitrogen fixation) [3]. Of particular importance to sustainable agriculture is the impact of salinity on the legume-rhizobia symbiosis. Although rhizobia are characterized by great variability in salt resistance [4], the selection of NaCl-tolerant strains, together with the fitness improvement of native strains under salt stress, can enhance the establishment of an effective rhizobium-legume symbiosis in saline soil [5] useful for the recovering of marginal areas. Thus, the investigation of the genetic basis of salt resistance in rhizobia is expected to contribute to the selection of symbiotic systems with adaptative potential to saline soils. In this work, a deep *in vitro* screening of salt tolerance of 48 *Sinorhizobium meliloti* strains, both Algerian isolates and collection strains, was performed on increasing concentration of NaCl. To identify genes that might be responsible for salt resistance in *S. meliloti* strains, we performed a *k*-mer-based GWA analysis using the tool PhenotypeSeeker. In particular, the genome sequences of the *S. meliloti* strains and the phenotypic matrix, gained with the *in-vitro* salt screening at 600 mM NaCl, were used to pinpoint specific *k*-mers significantly associated with salt-resistance phenotype. The retrieved *k*-mers allowed us to identify genes and candidate functions mostly involved in carbohydrate metabolism (specifically peptidoglycan transport and degradation, sorbitol biosynthesis, trehalose metabolic process, and cyclic-glucan synthesis) and galactose metabolism, but also in cell wall organization and LPS biosynthesis, quorum sensing, and DNA recombination and repair. This study will expand the knowledge of the mechanisms of salt resistance in *Sinorhizobium meliloti* and it may have a direct application in the selection of rhizobial inoculants for the improvement of alfalfa production in agricultural systems affected by salinity.

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AGRO-PHYSIOLOGICAL RESPONSE OF WHEAT AND FABA BEAN IN MONOCULTURE AND INTERCROPPING SYSTEMS TO PGPR-RHIZOBIUM CONTAINING CONSORTIA UNDER ABIOTIC CONSTRAINTS

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Drought and phosphorus (P) deficiency are one of the major challenges facing crop production woldwide. Intercropping systems showed positive facilitative interactions which may enhance microbial diversity, and plants nutritional status in terms of nitrogen and P. Inoculation with multi-strain bacterial consortia could stimulate the advantageous effects of intercropping system. However, little is known about the tripartite interaction between rhizobia, PGPR, and intercrop species, under stress combining drought and P-deficiency. Here, we evaluated the performance of three rhizobia-containing consortia (C_4, C_2 and C_{ref}) and single rhizobia inoculation on morpho-physiological parameters of intercropped and sole-cropped wheat and faba bean plants under P-deficient well-watered, or drought P-deficient treatments, versus a positive (Monoammonium di-phosphate) and a negative control. Our results showed that C4 is more likely adapted to well-watered P-deficient conditions. Indeed, shoot dry weight of intercropped wheat and faba bean reached up to 4.7 g. 4 plants^{-1} and 14.35 g. plants^{-1}, respectively. Faba bean nodulation was strongly enhanced following consortia inoculation under both water regimes compared to non-inoculated controls, with C_4 inducing the highest results. These results indicate that PGPR promoted nodulation by rhizobia. Also, inoculation with the rhizobia containing-consortia had a significant, variable response on morpho-physiological root traits shoot inorganic P content, and acid phosphatase compared to single rhizobia inoculation and controls. Additionally, consortia inoculation strongly increased above-ground physiological parameters, notably chlorophyll content, chlorophyll fluorescence, and plant leaf area compared to the negative control, whatever the cropping pattern or water regime. In conclusion, inoculation of intercropped and sole-cropped wheat and faba bean plants could be a promising solution to stimulate the growth of both intercrops under combined stress of drought and P-deficiency.

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NITROGEN FIXATION OF SOYBEAN AND LUPINE IN SWEDEN: DOES CULTIVAR MATTER?

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The cultivation of soybean and lupine in Europe has been on the rise in recent years. In Sweden, only a minor percentage of arable land is used to grow grain legumes. Colder and temperate climates, such as in Sweden, can limit the potential crop yield. Aside from climate, the presence of weeds can also act as a stress to the plant, for instance, by competing for resources. Stress has been shown to negatively impact nodulation in several legumes and actinorhizal plants. As a consequence of reduced nodulation, in terms of nodule size and number, it could also be expected that in such plants nitrogen fixation is also reduced.

We investigated the effect of climate and weed control on different cultivars of soybean, lupine, and faba bean grown in Sweden. Plants were grown in 2020 and 2021. The root systems of plants were collected, and nodulation was assessed based on total nodule weight, nodule number, and the individual nodule area. From the grains, the isotope ratios and mass fractions of C and N were measured, and the $^{15}N/^{14}N$ ratio was used to calculate how much nitrogen was fixed. Our results show that both the field site, which differed in local climate, and weed management affected nodulation, total nitrogen content, and amount of fixed nitrogen. Most importantly, different cultivars responded differently to these parameters. This indicates the importance of the choice of cultivar of soybean and lupine in organic farming.

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EVALUATION OF *SINORHIZOBIUM MELILOTI* ALGERIAN STRAINS FOR ALFALFA *IN VITRO* AND IN FIELD APPLICATION UNDER SALINE STRESS

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Degradation of soil quality due to drought and salinity stress is one of the most severe and widespread problems affecting agricultural productivity in arid and semi-arid areas [1]. The progressive salinization of soil strongly influences the symbiotic interaction between alfalfa and their associated rhizobia affecting the early stages of the symbiotic process. Successful symbiotic N_2 fixation under salt stress can be obtained if both partners resist such stress [2]. Generally, alfalfa cultivars are considered more sensitive to salinity than their rhizobial partner [3], highlighting how the choice of saline-tolerant cultivars is a key factor for high legume yield in saline soils [4]. However, the selection of NaCl-tolerant rhizobia is still fundamental for increasing alfalfa yield in saline conditions [5]. With this aim, extensive isolation and identification of *Sinorhizobium meliloti* strains from alfalfa root nodules in four different sites in Algeria were performed. Also, the efficiency of the symbiotic pairs *S. meliloti* - *Medicago sativa* was evaluated at 0 mM and 100 mM NaCl in controlled conditions. The strains able to efficiently improve plant growth at 100 mM are different and specific for each cultivar, highlighting how specificity and (microbial) G x (host) G interaction in this symbiosis has to be considered in the selection of the rhizobia inoculants. The obtained results allowed to identify 5 strains as candidate inoculants tested in a field more similar to natural conditions, with induced osmotic stress due to the absence of irrigation.

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DIVERSITY AND SYMBIOTIC PERFORMANCE OF *MESORHIZOBIUM* STRAINS COLLECTED IN CHICKPEA CROPPING AREAS OF AUSTRALIA AND MYANMAR

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The diversity of chickpea-nodulating Mesorhizobium identified in major chickpea producing countries may reveal greater diversity and assist in the development of new inoculants. Newly isolated strains collected from across Australian and Myanmar cropping regions were analysed in a pot experiment to evaluate nodulation and symbiotic effectiveness in chickpea. Phylogenetic analyses revealed chickpea nodulating rhizobia in Myanmar soils were most closely related to *M. gobiense, M. muleiense, M. silamurunense, M. tamadayense* and *M. temperatum*. Two-thirds of the Myanmar strains (68%) were most closely related to Indian strain IC-2058 (CA-181), which is also most closely related to *M. gobiense*. There were no strains that were closely related to the cognate rhizobial species to nodulate chickpea: *M. ciceri* and *M. mediterraneum*. This contrasts with Australian soils that were dominated by *M. ciceri*, *M. temperatum* and *M. huakuii*. The only co-occurring species found in both Myanmar and Australia were *M. tamadayense* and *M. silumurunense*. Continued inoculation with CC1192, which originated in Israel, may have reduced diversity of chickpea strains in Australian soils.

Strains from the above isolations were selected from symbiotic effectiveness studies conducted in the glasshouse and were tested in field trials in four southern Australian environments over two years. Strains isolated from Myanmar soils were inferior in nodulation compared to the Australian strains and CC1192. Strains collected from Australian soils had superior in survival on seed, improved nodulation and shoot dry weight at most experimental sites, compared with strains isolated from Myanmar soils. At most field sites, the newly isolated strains did not perform better than CC1192. Strain A47, collected in Australia, was the most effective of the strains tested in this study, with improved symbiotic N_2 fixation at one site. Australian strain A78 had equally effective nodulation as CC1192 on a highly acidic soil (pH 4.18 $CaCl_2$) at one site, while other test strains had inadequate nodulation at that site. Further testing may reveal environments where some of these strains exhibit greater soil adaptation and symbiotic effectiveness.

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SPATIO-TEMPORAL DYNAMICS OF NITROGEN FIXATION IN A LARGE LOWLAND RIVER WITH GEOMORPHIC COMPLEXITY

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The present investigation was carried out over 50 km reach the Padma River of Bangladesh, downstream of the confluence of the Ganges and Brahmaputra rivers. The study area is highly dynamic, with various kinds of geomorphic units, such as primary and secondary channels (C & S) islands (VI), bars (L, T and SB), vegetated bank (EK), dry channel (ED) and water depression (WD). A field study was carried out in low flow (dry/winter) season to measure the nitrogen fixation rate in each type of GUs. NFR in the C & S (water column) were upscaled using the monthly observed flow velocity, discharges in outflows and estimated water retention. And NFR in terrestrial GUs was upscaled in different seasons based on the surface area of GUs. Results showed that the highest NFR in C & S was obtained during monsoon due to the high volume of water. Among terrestrial GUs, the highest NFR was estimated in VI during dry/winter due to increased surface area. Later, Principal Component Analysis (PCA) was applied considering the seasonal variation of terrestrial GUs (surface area and number) and the distribution of nitrogen fixation estimated in GUs in different seasons. PCA showed that changes in the number and surface area of GUs across seasons could alter NFR. This systematic investigation of the spatial and temporal distribution of NFR in different GUs might be essential for estimating the nitrogen budget annually. Thus monitoring of NFR will help plan river restoration and ecosystem management programs.

Keywords: (Nitrogen fixation, geomorphic units, Seasonal variation, Tropical river, Bangladesh)

Md Ataul Gani is from Bangladesh and is registered as a fulltime PhD fellow in the Water Resources and Ecosystems Department of IHE Delft, The Netherlands. Since 2018, he has been researching the topic of “Nitrogen retention in different geomorphic units of a large lowland river in Bangladesh”.

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INFLUENCE OF WATER MANAGEMENT PRACTICES ON N_2 FIXATION IN RICE

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Flooded rice paddies are estimated to have the highest rates of nitrogen fixation among major cereal crops, where nitrogen fixation contributes an estimated 22kg ha^{-1} yr^{-1}. Both the paddy water and soil are home to free-living and associative diazotrophs capable of fixing atmospheric nitrogen. Yet, concerns over climate-induced water shortages and competition for existing water sources suggest that continuously flooded rice may be replaced by water saving alternatives such as alternate wetting and drying or aerobic cultivation in the future. If so, the removal of paddy water and alteration of soil oxygen availability due to these practices has the potential to impact rates of nitrogen fixation. Here, we use novel $^{15}N_2$ techniques to directly quantify how water management influences nitrogen fixation in rice.

The experiment utilized a fully enclosed greenhouse growth chamber (~1200 L). The chamber was designed to maintain $^{15}N_2$ gas enrichment levels (~3%) while also monitoring and controlling temperature, humidity, pressure, CO_2, O_2, and other trace gases. Treatments were carried out in 12x12x24 cm pots and included: continuous flooding, alternate wetting and drying (AWD) cultivation, and aerobic cultivation. Plants were grown outside of the chamber for 30 days before being transferred to the enrichment growth chamber until maturity. At harvest, soil cores (0-1 cm and 1cm+), plant biomass (roots, straw, and panicles), algae, and weeds were collected and processed. All samples were subsequently analyzed for total N and 15N ratios to calculate total fixed N and percent of N derived from the atmosphere (%Ndfa).

Across treatments, biological nitrogen fixation contributed between 3-12 kg N/ha to the system when extrapolated. On average, ~67% of the fixed N remained in the soil while ~30% was found in rice biomass. Aerobic cultivation reduced total fixed nitrogen, but AWD was not a significant effect. However, there was still a trend of decreasing water and decreasing rates of nitrogen fixation. This has implications for long-term nitrogen budgets as water shortages across global rice regions become increasingly common.

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PHR1 MODULATES ESSENTIAL GENES IN THE ROOT NODULE SYMBIOSIS

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Phosphate (Pi) homeostasis is tightly controlled by the transcription factor phosphate starvation response1 (PHR1) [1]. We recently demonstrated that PHR1 regulates the root nodule symbiosis by activating the Autoregulation of Nodulation (AON) pathway under Pi-deficient conditions[2]. New transcriptional evidence reveals that PHR1 modulates the expression of different symbiotic genes required to establish a successful root nodule symbiosis in common bean (*Phaseolus vulgaris*). This transcriptional data provides a new line of evidence supporting the notion that PHR1 is an essential regulator of the root nodule symbiosis in common bean.
In conclusion, we have demonstrated that PHR1 is an important genetic component of the root nodule symbiosis in common bean.

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N-FIX POTENTIAL OF SOIL MICROORGANISMS IN REDUCTION OF N FERTILIZATION IN AGRICULTURE

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Nitrogen is one of the most important elements of life and is one of the major limiting nutrients for most crop and other plant species. Simultaneously, the most common nitrogen component on earth – dinitrogen (N_2) – is not available for plants. In order to obtain satisfying amount of crop there is a need for nitrogen supplementation. Chemical fertilizers are more and more unwelcome. The alternative is nitrogen fixation carried out by free-living or symbiotic bacteria. The most efficient nitrogen fixing bacteria are *Rhizobium* species, which form an endosymbiotic nitrogen-fixing association with roots of (primarily) legumes and other flowering plants. On the other hand the important role in agriculture plays nonsymbiotic bacteria, that are highly diverse and globally widespread in cropland.

Presence of autochthonous, beneficial microorganisms is constantly under the pressure of abiotic stress and overuse of chemicals. Also effectiveness of nitrogen fixing is limited by various factors, among which there are availability of microelements as a part for nitrogenase complex (like Fe, Co or V), increased O_2 level or sub-optimal pH.

Here we discuss a potential of nitrogen fixing *Paenibacillus polymyxa* under different conditions and compare to other N-fix bacteria. Also, other biologically important features like mineral solubilization were evaluated.

The presence of biodiversity of microorganisms in the rhizosphere allow plants to reduce consequences of stress factors, like drought, non-optimal soil pH, extreme temperature and nutrient limitations. Also, allows plants to use fertilizers more effectively, and as a result acquires high crops of appropriate quality without the need for the excessive increase of the fertilization level.

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STABLE, BROAD HOST-RANGE FLUORESCENT MARKERS FOR TRACKING MULTIPLE BACTERIA ON PLANT ROOT

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Plant roots host a diverse range of microorganisms that offer mutual benefits[1]. Although microbiome studies usually involved counting and classifying these microbes using sequencing techniques[2], the cutting-edge challenge is to understand how a microbiome assembles. To simplify this process, a Synthetic Community (SynCom) is used.

We developed a Differential Fluorescent Marker (DFM) tool based on miniTn*7* transposon plasmids (pTn7-SCOUT) for modular assembly. It is based on the use of three fluorescent proteins (TagBFP, sYFP2 and mCherry), assembled in single or double combination. This allows us to quantify and differentiate up to six bacteria in a SynCom on plant roots using flow cytometry, making it independent of sequencing or culturing, and therefore less labour-intensive. The mini-Tn*7* transposon integrates into the chromosome, reducing the fitness effect and increasing the stability of the fluorescence expression cassettes[3]. Moreover, the integration site is highly conserved among bacteria, making the DFM tool broad-range tool.

We applied DFM tool to a SynCom (OxCom6) consisting of well-known root colonisers of Alpha- (*O. pituitosum* AA2, *R. leguminosarum* 3841), Beta- (*A. olearius* DQS-4, *A. xylosoxydans* AT1) and Gamma-proteobacteria (*E. cloacae* AA4, *P. fluorescence* SBW25), and studied their assembly pea and barley roots. After seven days, we observed a different SynCom assembly on each plant, where *P. fluorescence* SBW25 is the main coloniser on pea roots, whereas *E. cloacae* AA4 is on barley. Our results demonstrate that DFM tool is highly versatile and modular tool for tracking a diverse SynCom of six members on roots. Moreover, it can be applied to other types of communities consisting of different species, strains, or mutants, growing in free-living or in association with plants.

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IRON FERTILIZATION EFFECT ON N_2 FIXATION IN THE OCEAN DEPENDENT ON N_2-FIXING MICROBIAL COMMUNITY

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Biological nitrogen (N_2) fixation is the largest source of fixed nitrogen (N) to the ocean and therefore exerts an important control on ocean productivity. The high Fe requirement of the N_2-fixing nitrogenase and experiments on cultivated microorganisms indicate that Fe availability may control N_2 fixation activity in the ocean[1]. Furthermore, oceanic regions with high dust (i.e. Fe) fluxes harbor some of the highest N_2 fixation rates. There is, however, little evidence so far for a direct link between Fe input and N_2 fixation activity by microorganisms in the environment. We used stable isotope incubations (^{57}Fe and $^{15}N_2$) followed by single-cell uptake measurements (using nanoSIMS) to show that cellular rates of N_2 fixation are positively correlated with cellular Fe uptake in key N_2-fixing microorganisms in the ocean, *Trichodesmium* sp. and the symbiotic *Richelia*. Nevertheless, an increase in Fe availability did not necessarily lead to increases in N_2 fixation rates and sometimes even decreased N_2 fixation activity, possibly due to changes in resource allocation. The abundant and globally important *Trichodesmium* seemed to be most sensitive to Fe addition, which may have broad consequences for the productivity of the oligotrophic ocean and subsequent sequestration of atmospheric CO_2 to deep waters. The divergent responses of the different N_2-fixing microorganisms to Fe fertilization further indicate that global biogeochemical models may require prior knowledge not only of the structure of the N_2-fixing microbial community but also the degree of nutrient stress in order to predict N_2 fixation in the contemporary and future ocean.

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DECIPHERING COMPETITIVENESS OF ROOT COLONIZERS WITH THE USE OF SYNTHETIC COMMUNITIES

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Plant roots are home to a vast array of microorganisms, collectively known as microbiota. While many of these microbes have no discernible effect on the host, they play a crucial role in maintaining microbial homeostasis at the root-soil interface. By studying the genomes of entire microbial communities, rather than just individual strains, we can gain access to microbial genetic elements that inform how microbe-microbe and host-microbe- interactions establish. This approach allows us to identify genetic factors that contribute to niche supremacy, shedding light on the mechanisms by which symbionts and pathogens arise and how homeostasis can be maintained. We investigated this by inoculating complex but well-defined synthetic communities (SynCom) to *Arabidopsis*, *Lotus,* and barley roots in reconstitution experiments. These communities are taxonomically diverse and composed of rhizosphere-derived, full-genome sequenced bacteria (n=1200) isolated from these hosts grown in soil. Using metagenome sequencing, we identified a reduced number of isolates (n=40) that dominate the root niche in a host-specific or community-specific manner. Comparative genomics and microbial Genome-Wide Association Studies (mGWAS) of the competitive colonizers against the complex inoculum identified molecular functions associated with their colonisation behaviour. *Lotus japonicus* engages in root nodule symbiosis with *Mesorhizobium loti*. So far, most of the bacterial genes important for nitrogen-fixing symbiosis have been identified based on mutant analyses on single inoculations. Legumes, however, establish the symbiosis and their root microbiota concomitantly, but we lack knowledge of genes in the symbiont or microbiome that contribute to optimal symbiosis and beneficial host-soil microbe associations. Our comparative analyses identified bacterial genetic elements that are enriched in *Lotus*-compatible commensal bacteria and novel functions in symbiotic rhizobia compared to other, nonsymbiotic *Mesorhizobium* spp.. Together, our studies unravelled novel functions enriched during root nodule symbiosis in a community setting, providing a solid fundament for genetic studies to underpin their role in root nodule symbiosis in the field conditions.

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PROMOTION OF PULSE LEGUME INOCULATION IN AFRICA IN THE HealthyFoodAfrica CONSORTIUM

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HealthyFoodAfrica (HFA) is a research & innovation project aiming at more sustainable, equitable and resilient food systems in 10 African cities. The project is a collaborative effort by 17 partners in Europe and Africa, funded by the European Union Horizon2020 program. Our team at the University of Helsinki is leading the work package on sustainable food production. A survey of the sustainability of food production among our partners in Ethiopia, Ghana and Uganda targeting ecological, sociocultural and economical sustainability identified unsustainable practices that should be addressed. An important aim of ours is to promote legume inoculation as a sustainable and healthy practice in food production. There are very few rhizobium inoculant producers in Africa and those who are active face many obstacles that should be overcome. Inoculants are mostly of low quality and produced from exotic rhizobial strains. Factors hampering inoculant use are the insufficient microbiological skills of the personnel, the lack of quality culture collections, problems with scaling up and administrative obstacles. Effective N-fixing rhizobial inoculants of high quality are needed to increase biomass and grain yield of food legumes and to improve soil health, resilience and sustainability in food production. Grain legume inoculation trials with common beans and soybeans have been conducted since 2020 by partners in Tamale, Ghana, Rwamwanja refugee settlement and Fort Portal in Uganda, and Bahir Dar University, Ethiopia. Field trials in collaboration with farmers in the Bahir Dar area were performed with elite rhizobial strains for beans, isolated, characterized and tested in previous projects[123], and now used in inoculants produced locally. The production and training of local experts and lab technicians were done in collaboration with University of Helsinki. Makerere University produced inoculants and facilitated training for experts and model farmers in Uganda. In Tamale, soybean inoculated with locally produced inoculants gave higher yields in the farmers' fields than non-inoculated. In Bahir Dar, the performance of inoculated beans was remarkable and results from Bahir Dar will be presented. A model for inoculant selection and production will be displayed.

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ENCEPHALARTOS NATALENSIS, THEIR NUTRIENT-CYCLING MICROBES AND ENZYMES- A STORY OF SUCCESSFUL TRADE-OFFS

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Encephalartos natalensis is widely distributed in savanna woodland nutrient-poor ecosystem soils, and it is near threatened in the wild. Similar to other cycads, *E. natalensis* may thrive in the nutrient poor soils because of their symbiosis with nitrogen (N) fixing and other nutrient cycling bacteria. However, the identity of the *E. natalensis* associated symbionts with nutrient cycling functions and their contribution to soil fertility is not well understood. Due to *Encephalartos* spp. being threatened in the wild, this limited information presents a challenge in developing comprehensive conservation and management strategies for these cycad species. Hence, this study identified the nutrient cycling bacteria in *E. natalensis* coralloid roots, rhizosphere, and non-rhizosphere soils. Additionally, the soil characteristics and soil enzyme activities were assayed. The coralloid roots, rhizosphere, and non-rhizosphere soils of *E. natalensis* were collected from a population of >500 *E. natalensis* in a disturbed savanna woodland. Nutrient cycling bacteria such as *Lysinibacillus xylanilyticus*; *Paraburkholderia sabiae*, and *Novosphingobium barchaimii* were identified in the coralloid roots, rhizosphere, and non-rhizosphere soils of *E. natalensis*. The N-fixing microbes effectively fixed approximately 73% of the total plant N from the atmosphere. Phosphorus (P) cycling (alkaline and acid phosphatase) and N cycling (β-(D)-Glucosaminidase and nitrate reductase) enzyme activities showed a positive correlation with soil extractable P and total N concentrations in the rhizosphere and non-rhizosphere soils of *E. natalensis*. The positive correlation between soil enzymes and soil nutrients demonstrates that the identified nutrient cycling bacteria in *E. natalensis* coralloid roots, rhizosphere, and non-rhizosphere soils and associated enzymes assayed may contribute to soil nutrient bioavailability of *E. natalensis* plants growing in acidic and nutrient-poor savanna woodland ecosystems.

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ACTIVE NITROGEN FIXATION BY IRON-REDUCING BACTERIA IN RICE PADDY SOIL AND ITS ENHANCEMENT VIA IRON APPLICATION

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A large amount of nitrogen (N) fertilizer is currently applied to agricultural fields, which causes environmental problems associated with nitrogen pollution. Crop production with lower N fertilizer use is necessary and could be achieved by utilizing N-fixing microorganisms in agricultural soils. Our recent metatranscriptomic analysis and isolation studies discovered new N-fixing soil bacteria, *Anaeromyxobacter* and *Geobacter*, known as iron-reducing bacteria, which are predominant in paddy soils[1,2]. We hypothesized that adding ferric iron oxide as an electron acceptor for the respiration process of iron-reducing bacteria could enhance the N-fixing activity of these bacteria in paddy soils. In fact, soil N-fixing activity significantly increased after the addition of ferrihydrite or Fe_2O_3 to laboratory soil microcosms and iron powder to paddy field soils[3].

Here, with special reference to iron-reducing N-fixers, we performed ^{15}N-DNA-stable isotope probing (SIP) together with ^{15}N-IRMS analysis of native and iron-applied paddy field soils to (i) investigate the active N-fixing microorganisms and (ii) quantify the amount of fixed N. SIP analysis revealed that the most active N-fixers were *Anaeromyxobacter* and *Geobacter* in both native and iron-applied paddy soils. *Bacteroides*, known to have iron-reducing activity, was identified as another active N-fixing bacteria in the iron-applied soil. IRMS analysis revealed a significant increase in the amount of fixed N in the iron-applied soil. These results indicated that (i) *Anaeromyxobacter* and *Geobacter*, newly discovered iron-reducing N-fixers, are primary drivers of N fixation in paddy soils and (ii) iron application to paddy soil can increase the amount of fixed N in the soil by enhancing their N-fixing activity. This study may lead to novel and unique paddy soil management strategies to increase soil N fertility and to ensure rice yields with reduced N fertilizer inputs and lower environmental N burdens.

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SYMBIOTIC EFFICIENCY AND PGPR CHARACTERIZATION OF RHIZOBIA AND NON RHIZOBIAL ENDOPHYTES ASSOCIATED WITH CHICKPEA IN CROATIA

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The application of beneficial microorganisms (with emphasis on rhizobia and other PGP rhizobacteria) can significantly enhance agricultural sustainability and environment protection. The objective of the present study was (I) to assess phenotypic and PGP characteristics of chickpea isolates in laboratory conditions (II) to test the selected isolates for their symbiotic efficiency under field conditions. The results showed that all isolates grew well at elevated NaCl, temperatures and different pH (4 to 9). A total of 44 isolates were selected for PGP characterization. The ability to synthesize exopolysaccharides was determined within 37 isolates while only 11 and 8 were able to synthesize protease and amylase respectively. Phosphate solubilisation ability was found for 17 isolates and some non-rhizobia were characterized by high IAA production. Three different field trials were set up in 2022. In Porec (Institute of Agriculture and Tourism Porec, Istria) the highest values for both N content and seed protein content were obtained for plants inoculated with indigenous rhizobial strain 30b. In the experimental field of Faculty of Agriculture, Zagreb the chickpeas response to rhizobial inoculation was significant for almost all measured characteristics but the effect of intercropping, which was conducted with *Nigella*, was not determined. In the third location, family farm in Zadar, the application of PGPR strain had no significant effect on measured parameters. Inoculation with indigenous rhizobial strain 47h increased seed yield and seed protein content but the presence of indigenous rhizobial population in the soil limit the success of inoculation. As different values for seed yield were determined depending on location and application of particular strain, further investigation is needed in order to select rhizobial strains with enhanced symbiotic efficiency which will enable reduction of mineral fertilizers and impact of unfavourable environmental conditions.

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IMPACT OF SEED-APPLIED FUNGICIDES ON RHIZOBIAL SURVIVAL IN CHICKPEA

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The addition of seed-based fungicides to pulse seeds before sowing is a recommended practice in Australian cropping systems. Chickpeas have a particular susceptibility to *Ascochyta* infection and pulse growers are advised to be treated seed with a thiram-based fungicide to prevent disease. Rhizobial peat-based inoculant is usually coated over this fungicide, to ensure effective rhizobia are present to nodulate chickpeas. However, thiram has a toxic effect on *Mesorhizobia ciceri* strain CC1192, the commercial inoculant used in Australia (Rathjen et al 2020). Although the survival of CC1192 in the laboratory and nodulation in the glasshouse can be reduced, there is very little evidence of the effect of this chemical on rhizobial survival in a competitive soil environment in the field.

We conducted five field trials over two years to determine the effect of thiram-based fungicide on the nodulation and performance of chickpea plants. Plants that had seeds coated with thiram before peat inoculation had lower nodulation and N fixation than control plants not coated with the fungicide. To determine if the toxicity of the fungicide could be avoided by separation from the inoculant, we used granular inoculant with coated seeds. The plants inoculated with granules showed improved nodulation compared with the control. However, separation with a liquid-based inoculant did not prevent reduced nodulation associated with the thiram. Despite this, yield was not affected significantly in most sites, and in one site was increased in plants with seeds coated with thiram.

Although thiram-based fungicides negatively affect rhizobial survival, fungicides with metylaxyl as the active ingredient did not impact the growth of CC1192. We hypothesised that these fungicides may promote root growth under high disease prevalence, thereby enhancing nodulation and N fixation through improved root hair production. In four field trials conducted over two years with three different fungicides it was found that, in general, the metalaxyl fungicides had a positive effect on nodulation. This varied between sites and fungicides and may have been due to the presence of different root disease pathogens.

Even though seed-applied thiram causes a decline in nodulation in chickpea plants, farmers are faced with the challenge of requiring *Ascochyta* control as well as an effective inoculant. We have explored creating a thiram tolerant mutant of CC1192 which could be used safely with fungicides as a peat inoculant without the risk of using alternative inoculant types. CC1192 mutants have shown improved seed survival on seeds coated with thiram and are in the process of being evaluated in glasshouse experiments.

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SYMBIOSIS WITH NEW LEGUME PLANTS FOR CLIMATE CHANGE MITIGATION IN NORTH-EAST EUROPE

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In the current European cropping systems only 1.5% of arable land is dedicated to cultivating legumes. It leads to a deficit of 70% of high-protein crop commodities for animal feed in Europe. This way current food chains are unsustainable from consumers, producers, climate and the environment points of view. Our research aims to investigate if diversification of crop rotation by new legume crops such as soybean, lentil and chickpea can advance the implementation of Green Deal goals, such as climate change mitigation and adaptation and what role in it has plant-specific symbiotic and endophytic bacteria.

Our results showed that these legumes might be a good tool to ensure food security in the face of climate change. With increased co-occurrence of drought and uneven moisture during the growing season, soybean, chickpea and lentil compete well in productivity with traditional legume plants - field pea and fababean. Most competitive in grain yields with local legume species was soybean, and least competitive was lentil. Using symbiotic bacteria ensured effective N fixation from the atmosphere for soybean and chickpea. Isotope based quantification of nitrogen fixation in symbiosis with different microorganisms gave important tender to climate change mitigation. New legume plants fixed from 26 to 175 kg N ha^{-1} depending on the microorganism used. Also, soybean was shown to have equal or higher rotational residual effect on subsequent cereals as field pea, than using different cultivars of Bradyrhizobium japonicum. However, these plants had their own risks, such as logging for lentil, diseases for chickpea and prolonged vegetation for soybean, which could be solved by genotype selection. Our studies give the lists of advantages and disadvantages of cultivating these plants in North-East Europe and also presents the symbiotic bacteria cultivars for effective nitrogen fixation.

Keywords: soybean, lentil, chickpea, Rhizobium, diversification

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UNCOVERING MICROBIOME ASSOCIATED WITH WINTER PEA NODULES

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Rhizobia are not the only inhabitants in legume nodules. Other nodule-occupying bacteria are known to originate from the soil and interact with the nodule microbiome to affect N fixation and fitness of the host plant. Additionally, little is known about the composition of the nodule-occupying fungal community and its role in establishment and maintenance of effective rhizobium-legume symbiosis. In this study we assessed the composition of the bacterial and fungal root and nodule microbiome associated with 4 food grade winter pea, *Pisum sativum L.*, cultivars across 3 fields in Washington state. Tissue type (nodule *vs.* root) and field location were the most significant factors effecting the community. Several fungal genera, including Didymella and FunneliformisI, were differentially represented between the root and nodule microbiomes or between cultivars. *Rhizobium* was the only genus significantly overrepresented in the nodules compared to the roots. We identified a single major *Rhizobium* amplicon sequence variant (ASV) occupying both roots and nodules of plants grown in all locations, and overrepresented in the nodules. A limited number (<10) of *Rhizobium* spp. ASVs were unique to nodule tissue and differed between locations. Most of these ASVs were shared between nodules from plants grown at the same location. Additionally, based on PacBio sequencing, two *Acidiphilium* ASVs, *A. multivorum* and *A. acidophilum*, were uniquely identified in the nodules at one of the locations. This data indicates that, while Washington soils share a common highly competitive winter pea rhizobial symbiont, they also contain symbionts unique to specific locations. This data also suggests that fungal community is part of the complex interaction between a N-fixing legume-host and the soil microbiome and its role in establishment of effective symbiosis should be further investigated. The cultivar genotype should be also considered as a factor affecting legume-microbiome interaction.

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ROLE OF BACTERIA-DERIVED FLAVINS IN VEGETABLE GROWTH PROMOTION

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Riboflavin, commonly known as vitamin B2, is an essential element of living organisms and is the precursor of flavin (FLs) cofactors, flavin mononucleotide and flavin adenine dinucleotide. *Sinorhizobium meliloti* 1021 establishes a nitrogen-fixing symbiosis with Medicago plants and secretes considerable amount of FLs (FL+). This strain was also implicated in plant growth promotion in its association with non-legume plants. However, the mechanism of this plant growth promotion is not well understood. In this study, we tested our hypothesis that bacteria derived FLs are involved in the ability of *S. meliloti* 1021 to promote host-plant growth. We evaluated the growth and development of lettuce (*Lactuca sativa*) and kale (*Brassica oleracea*) plants inoculated with *S. meliloti* 1021 and its mutant 1021Δ*ribBA* with limited ability to secrete FLs (FL^-). Our results indicated that inoculation with FL^+ *S. meliloti* 1021 significantly increased the length and surface areas of root and hypocotyl of the seedlings compared to the inoculation with FL^- 1021Δ*ribBA*. For example, for the kale and lettuce, respectively, we observed 19% and 14% increase in total root lengths of seedling inoculated with *S. meliloti* 1021 compared to that inoculated with 1021Δ*ribBA* mutant. Greenhouse trial showed that total phenolics content and total flavonoids of plant leaf tissues were significantly increased with bacterial FLs secretion (Fig. 1, A and B). Moreover, the inoculation with *S. meliloti* 1021 apparently improve plant growth compared to the inoculation with 1021Δ*ribBA* (Fig. 1, C and D). Our data indicate that the ability to secrete FLs might contribute to bacterial plant growth promoting functions.

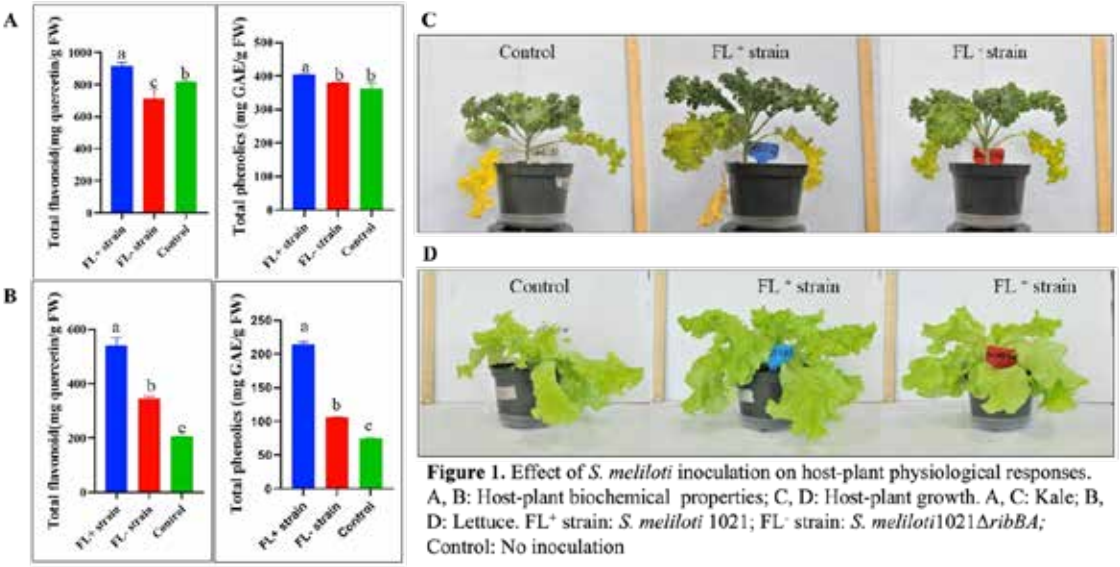

Figure 1. Effect of *S. meliloti* inoculation on host-plant physiological responses. A, B: Host-plant biochemical properties; C, D: Host-plant growth. A, C: Kale; B, D: Lettuce. FL^+ strain: *S. meliloti* 1021; FL^- strain: *S. meliloti*1021Δ*ribBA;* Control: No inoculation

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