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Differentiation of symbiotic nodule cells and their rhizobium endosymbionts

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Abstract

The symbiotic nodule cells are at the heart of the rhizobium-legume symbiosis. They harbor thousands of nitrogen-fixing rhizobia, called bacteroids, within symbiosome vesicles. The symbiotic cells differentiate from meristem cells and this process is driven by endoreduplication and a transcriptome switch, establishing an optimal environment for the maintenance and the metabolic integration of the nitrogen-fixing bacteroids. The differentiating symbiotic cells uptake rhizobia by an endocytosis-like process and the resulting symbiosomes are matured by a specific exocytotic pathway. Finally, the rhizobia within the symbiosomes differentiate into bacteroids involving a metabolic transition controlled by the low oxygen conditions in the symbiotic cells. Depending on the host plant, the bacteroid differentiation involves in addition an irreversible morphological and cytological transition, which is driven by host-produced peptides, called Nodule-specific Cysteine-Rich peptides or NCRs. The parallel differentiation of the host cells and their bacterial endosymbionts results in a unique cellular configuration that is extremely efficient for symbiotic nitrogen fixation.

Key words: symbiotic nodule cell; cell cycle; endoreduplication; endocytosis; exocytosis; symbiosome; bacteroid; NCR

Introduction

In response to nitrogen starvation and the presence in the nearby soil environment of specific compatible nitrogen-fixing rhizobium bacteria, legumes will engage into a symbiosis with these bacteria. The plants will form symbiotic organs on their roots, called nodules which are infected by the microsymbionts (Fig. 1). In the nodules, the rhizobia form in large quantities ammonium from nitrogen gas and transfer this reduced form of nitrogen to the plant that incorporates it in its metabolism and uses it for growth. Nodulated legumes obtain most, if not all, of their nitrogen needs from their nitrogen-fixing symbionts. Nitrogen is one of the major elemental constituents of plant biomass, fourth after carbon, hydrogen and oxygen (about 1-2% of the dry biomass is nitrogen, compared to 20-40% of carbon). Thus to sustain plant growth, the symbiosis must be capable of providing massive amounts of reduced nitrogen. This is achieved by producing large numbers of nodules in which very high amounts of bacteria are maintained (Fig. 1). At the heart of the nodules are the symbiotic nodule cells. These cells carry intracellularly the nitrogen-fixing rhizobia, called bacteroids (Fig. 1). A typical nodule will contain several thousands of symbiotic cells (Fig. 1B) and each symbiotic cell is infected with several thousand bacteroids (Fig. 1E). Thus, each nodule contains a few tens of millions of bacteria, the equivalent of a large plate-grown colony.

The heavily infected symbiotic nodule cells are among the most distinguishing features of nodules. Their formation, and also the overall nodule organogenesis, is based on similar processes in different legume lineages but it also diverges in quite some details. Here, I focus on the formation of symbiotic nodule cells in *Medicago truncatula* and closely related legumes of the Inverted Repeat-Lacking Clade (IRLC) but comparison with other legumes is made when relevant. In *M. truncatula*, the symbiotic nodule cells are formed from dividing nodule primordium cells or nodule meristem cells. Primordium or meristem cells that engage into the symbiotic cell formation pathway stop dividing and undergo endoreduplication that drives the differentiation process. Specific structures called infection threads conduct the rhizobia, starting from root hairs in the root epidermis, inside the tissues of the growing nodule (Fig. 1C) and release rhizobia inside the differentiating symbiotic cells through an endocytotic process (Fig. 1D). The endocytotic uptake does not release rhizobia freely in the cytosol of growing symbiotic cells but inside organelle-like vesicles, called symbiosomes. Within the symbiosomes, the rhizobia grow and differentiate into bacteroids, the nitrogen-fixing form of the rhizobia. This latter process is enabled by the exocytosis pathway, which traffics specific proteins to the symbiosomes. The maintenance of thousands of bacterial endosymbiont-containing symbiosomes within individual plant cells necessitates the acquisition of a specific vesicular identity of the symbiosomes and avoidance of a vacuolar identity that would lead to the elimination of the symbiont. In addition, the symbiotic cells have a specific machinery that suppresses innate immunity responses against the large number of endosymbionts within them. The host cell-endosymbiont homeostasis also involves the production of symbiosis-specific peptides called Nodule-specific Cysteine-Rich peptides (NCRs), which target the bacteroids and keep them under check by forcing them into a terminally differentiated state. Finally, the functioning of the symbiotic cells requires the integration of the bacterial metabolism with the metabolism of the host cells. Major features of the metabolism of the symbiotic cells is the supply of carbon derived from shoot photosynthesis to the energy-devouring bacteroids, the maintenance of a low oxygen concentration to avoid the irreversible oxidation of the nitrogenase enzyme and the assimilation of ammonium provided by the nitrogen-fixing bacteroids. The plant forces the bacteroids to uncouple nitrogen-fixation from the bacterial nitrogen stress metabolism so that the rhizobia generate ammonia in large excess to their own needs, contrary to what they would do as autonomous bacteria. They release this ammonia to the benefit of the plant. This specific metabolism requires the transcriptional activation of a suite of enzymes and transporters and the massive production of leghemoglobin to bind oxygen. This general outline of the symbiotic nodule cell formation will be

described in detail in the following paragraphs in which I will try to provide an integrated view of the differentiation of the symbiotic nodule cells, the symbiosomes and the bacteroids they contain.

Endoreduplication and infection driven differentiation of the symbiotic cells

Nodules are made *de novo* from fully differentiated root cells that, following the recognition of the rhizobial Nod factor signal, dedifferentiate and engage into the new developmental program of nodule organogenesis. In *M. truncatula*, the radial organization of roots consists, from the outside to the inside, in an epidermal cell layer, five cortical cell layers named C1 to C5, the endodermal cell layer, the pericycle cell layer and the vascular tissue. Based on sequential longitudinal sections of roots and nodule primordia, on the use of marker genes that allow the distinction of cells originating from different root tissues and on the use of nodulation mutants, a nodule cell fate map was constructed that precisely describes the origin of the different nodule tissues (Fig. 2A) (Xiao et al., 2014). This analysis showed that upon perception of the rhizobia and the initiation of infection in the epidermis, the first cell divisions occur in the pericycle and then spread into the cortex. Divisions are first anticlinal (perpendicular to the root axis adding cells within the same cell layer) and then, periclinal divisions happen that add new cell layers provoking the outgrowth of the incipient organ or primordium. While these divisions are ongoing, the infection threads cross the epidermis and the outer cortex and ramify as the branches of a tree. The C4 and C5 have formed about eight cell layers when the infection thread network reaches them and releases rhizobia in them. At this point, these cells stop dividing, switch to an endoreduplication cycle, and start the symbiotic cell differentiation process. The pericycle and endodermis have formed six to eight cell layers but these cell layers do not become infected. C3-derived cells that have not been infected form a multi-layered meristem. Next, the meristem starts to add cells to the nodule tissues, which on their turn become infected by infection threads and released rhizobia, upon which they switch to the endoreduplication-driven differentiation process. Several vascular bundles are established at the periphery of the incipient nodule, starting from the pericycle and endodermis cell layers. From this stage, the primordium has become a nodule. Thus in a mature nodule, the six to eight uninfected basal cell layers are derived from the pericycle and endodermis. The about eight most basal infected cell layers are derived from the C4 and C5 root cortical cell layers. The remaining infected cell layers are derived from the indeterminate nodule meristem and continuously increase in number with the nodule age due to the persistent meristematic activity (Fig. 2A).

Three-dimensional reconstructions of infection threads inside *M. truncatula* showed that the transition from a primordium stage to a mature nodule is also reflected in the architecture of the infection thread network (Monahan-Giovanelli et al., 2006). The infection threads form tree-like networks in both primordia and mature nodules. These infection thread networks have relative constant characteristics in terms of branch lengths and branch density in nodules of different developmental stages but the overall direction of growth of the networks changes as nodules develop. In primordia, the network grows from the epidermis toward the inner C4- and C5-derived cell layers. However, sub-regions of this network are repolarizing their direction of development in response to the establishment of the nodule meristem. In mature nodules, the network is entirely reoriented and its branches grow now toward the meristem and away from the nodule base.

The central tissues of a mature *M. truncatula* nodule is usually described as partitioned in distinct zones: zone I is the meristem; zone II, derived from the meristem, is the symbiotic cell differentiation and infection zone where the infection thread network grows; and zone III is the fixation zone where the symbiotic cells are mature. The zone III is thus in fact composed of cells with two distinct origins, the most proximal cells are derived from the C4 and C5 cell layers of the primordium and the more distal cells are derived from the zone II (Fig. 2A) (Xiao et al., 2014).

The nodule meristem is organized in at least two subdomain types, a central meristem and several peripheral vascular meristems in which different sets of marker genes are expressed and in which the levels of the auxin and cytokinin phytohormones are distinct (Franssen et al., 2015; Magne et al., 2018). These subdomains give rise to the different tissues and cell types that compose the nodule. Cells located at the edge of the nodule meristem engender the peripheral tissues of nodules, the parenchyma, endodermis and cortex, and the vascular meristems generate vascular bundles that are located in the nodule parenchyma and that surround the central nodule tissue like the fingers of a hand holding a ball. The central meristem is the source of the symbiotic cells that constitute together with specialized uninfected cells the central zone of the nodules. The differentiation into symbiotic cells requires that the cells at the edge of the central meristem stop dividing and switch the mitotic cell cycle into an endoreduplication cycle, a modified cell cycle in which no mitosis and cytokinesis is taking place but repeated genome duplications are happening, forming polyploid cells (Fig. 2B) (Vinardell et al., 2003). Endoreduplication is triggered very early on, as soon as bacteria are released in the cells (Vinardell et al 2003; Maunoury et al., 2010). The signal that triggers the cell cycle switch is unknown but the analysis of mutants in both *M. truncatula* and *Sinorhizobium meliloti*, its rhizobium symbiont, suggest that the trigger is related to infection. Indeed, the *vapyrin* and *lin* *M. truncatula* and *exoY* *S. meliloti* mutants form nodule-like structures without endoreduplication and in which infection threads are arrested in the epidermal cell layer (Maunoury et al., 2010; Guan et al 2013; Xiao et al 2014). Remarkably, the nodule-like structures formed by these mutants have a central vasculature instead of the normal peripheral bundles. Thus, it is as if the differentiation of the symbiotic cells inhibits the initiation of a vascular bundle in the pericycle and endodermis cells located immediately beneath them and shifts the vascular bundles to the periphery. At a later stage, in mature nodules, the NOOT1 and NOOT2 transcriptional regulators control the interaction between the central nodule meristem and the vascular meristems. The central meristem vanishes in the absence of these regulators and ectopic roots arise from the nodule vascular meristems (Magne et al., 2018). Another *M. truncatula* mutant, affected in the *IPD3* gene, connects the cell cycle switch of differentiation with the infection process. This mutant can form nodules with a meristem, peripheral vascular bundles and an extensive network of infection threads in its central zone without however releasing bacteria from them in the nodule cells. The cells of the central zone of *ipd3* nodules do not display endoreduplication either and are mostly in the 4C stage (four genome complements). Thus, in these nodules the central zone cells are arrested in the cell cycle but do not progress into an endoreduplication cycle. These cells do also not activate the characteristic transcriptome of the symbiotic cells (Maunoury et al., 2010). It seems thus from this, that penetration of an infection thread arrests division of meristem cells and that the endocytotic release of rhizobia is the trigger for endoreduplication, differentiation and the characteristic gene expression of symbiotic nodule cells. Some cells originating from the meristem in wild type nodules are not penetrated by the infection threads and thus never become infected by the rhizobia. These cells do not go into the endoreduplication cycles and can be seen as small cells in-between the large infected cells in the zone III (Fig. 1B). These uninfected cells have a specialized function in the metabolite transport to and from the infected cells (Limpens et al., 2013).

At the molecular level, several actors in the process of endoreduplication and endocytotic uptake have been identified. One of them is the cell cycle switch protein CCS52A. Master regulators of the eukaryotic cell cycle are cyclins that are active in different phases of the cell cycle and promote the transitions from one phase to the next. CCS52A is an activator of the anaphase promoting complex, an E3 ubiquitin ligase that targets cyclins for degradation by the 26S proteasome. CCS52A is expressed in the post-meristematic cells and promotes the degradation of mitosis-stimulating cyclins, thereby forcing the ongoing cell cycle in these cells into the endocycle (Fig. 2B) (Vinardell et al., 2003). When the *ccs52A* gene is knocked-down in nodules, the symbiotic cell formation is drastically affected, with

lower ploidy levels, reduced cell size, inefficient invasion, and no maturation of bacteroids. Thus, endoreduplication is an integral part of normal symbiotic cell formation.

The so-called common symbiosis signaling pathway (CSSP) is a module of regulators that control the initial stages of nodulation (Geurts et al., 2016). This module makes the plant response to the bacterial Nod factor signals possible in the epidermal root cells and is composed of a receptor complex and a signal transduction cascade that activate a downstream transcription factor (TF) network. Interestingly, using RNAi knock-down strategies that leaves the functioning of the *SymRK* and *NFP* components of the Nod factor receptor complex of the pathway unaffected in the root epidermis, allowing nodules to form, but that reduces strongly the activity of the genes at later stages, shows that the CSSP is required for the endocytotic uptake of rhizobia in the symbiotic cells (Limpens et al., 2005; Moling et al., 2014). Moreover, as mentioned above, mutants in the *IPD3* gene, encoding a TF of the pathway, can also not release rhizobia in the post-meristematic cells. The *ipd3* mutation does not block the epidermal function of the CSSP because of the presence of a second *IPD3* gene in *M. truncatula* that is sufficient for epidermal signaling but not for rhizobium uptake (Jin et al., 2018). From these observations follows that Nod factors produced by rhizobia in the infection threads activate the CSSP to trigger rhizobium uptake (Moling et al., 2014). Thus, the CSSP seems to be a multipurpose regulatory module that is used to control nodulation in different stages of nodulation, including the first responses to Nod factors in the root epidermis and the endocytosis of rhizobia in the infection zone of the nodule adjacent to the meristem.

As discussed in detail in the next paragraph, the endocytotic release of rhizobia further requires exocytotic vesicle trafficking to the site of infection as well as a specific architecture of the actin cytoskeleton for the guidance of the vesicles. Exocytosis and the actin cytoskeleton are subsequently also primordial in the maturation of the symbiosomes. The exocytosis process and the actin cytoskeleton could constitute a link between infection and the cell cycle switch. The cell division, and in particular the cell plate assembly, as well as the symbiosome formation and maturation depend on the exocytosis pathway (Kanazawa & Ueda, 2017) and at least in part on the same protein players, rendering these processes possibly incompatible with each other. In addition, the actin cytoskeleton architecture required for rhizobium uptake and subsequent symbiosome maturation on the one hand and for cell division on the other hand might also be incompatible with each other. Thus, possibly cells cannot execute simultaneously division and symbiosome formation. However, in certain legumes as for example in *Aeschynomene* spp., nodule founder cells in the root cortex are infected by direct uptake of intercellular rhizobia, without the involvement of infection threads. These infected cells subsequently divide repeatedly to form the symbiotic nodule cells of the central zone of the nodules (Bonaldi et al., 2011). This infection process is thus fundamentally different from the infection thread-dependent process. It is further interesting to note that these symbiotic nodule cells in *Aeschynomene* spp. are not endoreduplicated.

Exocytosis pathway in symbiotic nodule cells

Infection threads are transcellular structures, delineated by a plasma membrane, which is aligned in its interior by a cell wall and a matrix imbedding the rhizobia. To release bacteria inside a symbiotic nodule cell, unwallied infection droplets containing rhizobia are budding off next to the tip of an infection thread (Fig. 3). These infection droplets release rhizobia enclosed in the symbiosome vesicles that have a membrane derived from the plasma membrane. Therefore, the interior of infection threads and symbiosomes are equivalent to the apoplastic compartment despite the intracellular localization of these structures. Bacteria are repeatedly released in each symbiotic nodule cell, the symbiosomes initially also divide and in addition, they strongly enlarge before becoming mature (see below). The outcome is a symbiotic nodule cell that is completely filled with radially aligned symbiosomes (Fig. 1E).

Thus, the proliferation of the symbiosomes in a symbiotic nodule cell is so extensive that it requires considerable cellular investment in trafficking proteins and lipids to these organelles. Circumstantial evidence strongly suggested that the secretory pathway orchestrates this massive colonization of the symbiotic nodule cells by the microsymbionts. Electron microscopy imaging for instance shows the abundant presence of endoplasmic reticulum (ER), Golgi bodies as well as small vesicles associated and fusing with symbiosomes (Robertson & Lyttleton, 1982; Hirsch et al., 1983). Fluorescence immunolocalization of the ER shows a very strong signal in the developing and young symbiotic cells (Maunoury et al., 2010). Transcriptome analysis shows furthermore that symbiotic cells express a very high number of secretory proteins (Maunoury et al., 2010). Together, these observations show thus that the symbiotic nodule cells have a very strong secretory activity targeting the symbiosomes. Genetic and reverse genetic experiments combined with cell biology analyses reinforce this assumption and highlight the essential functions in symbiosome formation and maturation for multiple proteins of the secretory pathway (Fig. 3).

One of the first steps in handling secretory proteins is the cleavage of their signal peptide sequence in the ER by the signal peptidase complex (SPC) (Fig. 3). A forward genetic screen identified the mutant *dnf1* (*defective in nitrogen fixation 1*) of *M. truncatula* forming non-functional nodules (Wang et al., 2010). *DNF1* encodes SPC22, one of the subunits of the SPC. The *dnf1* mutation only affected nodule formation and no other phenotypes in the mutant plants were reported, despite the fact that the SPC is essential for viability in eukaryotes. The reason is that a second SPC22-encoding gene is present in the genome. Two additional components, SPC12 and SPC25, are also encoded by duplicated genes. Strikingly, *DNF1* and one copy of the SPC12- and SPC25-encoding genes are nearly exclusively expressed in nodules and are specifically active in the infection zone where symbiosomes are formed and grow. The other gene copies on the other hand have a ubiquitous expression explaining the absence of additional phenotypes in *dnf1*. The nodules of the *dnf1* mutant initially develop normally and release bacteria in symbiotic nodule cells, which become nearly completely filled with symbiosomes. However, these symbiosomes and the bacteria they contain remain unmodified and do not differentiate into their enlarged nitrogen-fixing form. At the molecular level, secretory proteins that are targeted to the symbiosomes in the wild type nodules, retain their signal peptide and are trapped in the ER in the *dnf1* nodules (Van de Velde et al., 2010). The *dnf1* mutant provides thus a genetic proof that the secretory pathway is crucial for the formation of functional symbiotic nodule cells in *M. truncatula*.

Additional genes encoding ER-localized components of the secretory pathway are upregulated in nodules and are co-expressed with the SPC genes in the infection zone of nodules (Maunoury et al., 2010). Among them are a gene encoding a signal peptide peptidase (SPP), an enzyme that degrades signal peptides after they have been cleaved off by the SPC; a protein disulfide isomerase (PDI) and ER oxidoreductin 1 (Ero1), both involved in the introduction of disulfide bridges in secretory proteins; a small family of nodule-specific secretory prolyl-4-hydroxylases possibly involved in modifications of prolyl residues of secretory proteins; and an ER lumen protein retaining receptor for sorting and retaining ER luminal proteins.

Exocytotic trafficking of proteins through the ER might have multiple final destinations, including the plasma membrane and the extracellular space, vacuoles, and in the symbiotic cells, thus also the symbiosomes. How the endomembrane trafficking process of a cell in general distinguish between possible destinations is determined by membrane identity markers that mediate specific vesicle delivery to corresponding target membranes. These proteins include the exocyst tethering factors and Rab (Ras-associated binding) GTPase proteins that promote the complex formation between vesicles and target membranes, and v-SNARE (Soluble N-ethylmaleimide-sensitive-factor Attachment protein REceptor) and t-SNARE complexes that mediate the fusion itself of the vesicle membrane with the

target membrane, resulting also in the delivery of the vesicle cargo into the target compartment. Symbiosomes must thus have specific factors that contribute to the exact delivery of secretory proteins.

The *M. truncatula* *Syntaxin 132* (*SYP132*) gene encodes two isoforms of the t-SNARE protein SYP132, resulting from alternative splicing (Pan et al., 2016; Huisman et al., 2016). One of these transcripts, *SYP132C*, has a ubiquitous expression while *SYP132A* is most strongly expressed in symbiotic nodule cells. Both protein isoforms are nearly identical and differ only in their last 60 amino acids. The *SYP132C* isoform is mostly found on the cell periphery, in agreement with the known plasma membrane localization of the SYP132 t-SNARE in plants. In contrast, *SYP132A* is localized to the symbiosome membrane (Fig. 3) (Pan et al., 2016; but see also Huisman et al., 2016). RNAi experiments specifically targeting one or the other alternative transcript revealed that *SYP132C* is essential for plant growth while the knock-down of the *SYP132A* transcript resulted in the formation of symbiotic cells containing undifferentiated bacteroids, a phenotype very similar to the *dnf1* mutant. Presumably, the lack of *SYP132A* on the symbiosome membrane precluded vesicle fusion and thus provoked a blockage in the transport of the nodule-specific secretory proteins to the symbiosomes needed for symbiosome and bacteroid maturation.

Also on the side of the vesicles that target the symbiotic compartment, key v-SNARE proteins have been identified. In plants, v-SNAREs belong to the VAMP72 family. By genome mining seven VAMP72 proteins were identified in *M. truncatula* and phylogenetic analysis comparing the family in *M. truncatula* with the corresponding family in the non-symbiotic Arabidopsis plant combined with transcriptional analysis, restricted the candidate VAMP72s to the closely related VAMP721d and VAMP721e (Ivanov et al., 2012). Contrary to the *dnf1* mutant and *SYP132A* knock-down, knock-down of the *VAMP721d* and *VAMP721e* genes blocked nearly completely symbiosome formation. Ultrastructural analysis showed that this was because infection droplets in these RNAi lines contained a cell wall, hampering the release of the bacteria from infection droplets (Ivanov et al., 2012). Probably this is the consequence of the inability to deliver VAMP721d/e-vesicles containing cell wall degrading enzymes to the droplets (Gavrin et al., 2016). Immunolocalization and confocal or transmission electron microscopy further showed that the VAMP721d/e proteins are located on vesicles that are in the vicinity of infection droplets as well as symbiosomes, consistent with a targeted VAMP721d/e-mediated delivery of exocytotic vesicles to droplets but also to symbiosomes (Fig. 3). Interestingly, MtVAMP721d/e accumulated at the site of cell plate formation in meristem cells, in agreement with the earlier proposed common machinery for cytokinesis in dividing cells and for infection in symbiotic nodule cells (Kanazawa & Ueda, 2017).

Strikingly, VAMP72a, closely related to VAMP72d/e, is repressed in symbiotic nodule cells thanks to the symbiotic cell-specific transcriptional repressor RSD (Sinharoy et al., 2013). This repression is essential for the symbiosome maintenance because in an *rsd* mutant, which has elevated *VAMP72a* expression in the symbiotic cells, bacteroids are not fully developed and strikingly, strong defense reactions are induced against them. Possibly, VAMP721a-bearing vesicles contain cargo different from that of VAMP721d/e-vesicles, and their fusion with symbiosomes in the *rsd* mutant delivers compounds that mount an immune response against the bacteria. In line with this hypothesis, Arabidopsis VAMP721/722, the closest homologs VAMP721a, are part of the secretory apparatus that targets vesicles containing defense compounds to the plasma membrane at sites of fungal pathogen infection (Kwon et al., 2008).

Other characterized components involved in vesicle fusion on the symbiotic interface are the calcium-sensing synaptotagmins that regulate the calcium-dependent vesicle fusions mediated by v-SNAREs and t-SNAREs (Fig. 3) (Gavrin et al., 2017). The *M. truncatula* synaptotagmins were localized nearby

the tips of infection threads, unwalled droplets and symbiosomes and their knock-down by RNAi retarded rhizobium release and blocked symbiosome maturation. The Rab GTPase Rab7 is also located on symbiosomes and could thus be involved in the tethering of secretory vesicles to the symbiosomes. Accordingly, knock-down of *Rab7* gene expression hampers symbiosome and bacteroid differentiation (Limpens et al., 2009).

In summary (Fig. 3), in the infection process of the symbiotic cells, the v-SNAREs VAMP72d/e are required before SYP132A and Rab7, for the formation of unwalled droplets but subsequently all three proteins cooperate in the focal vesicle delivery to symbiosomes. Thus, the t-SNARE and Rab proteins on the infection droplet remain to be described and are likely to be different from SYP132A and Rab7. Synaptotagmins are likely involved in regulating the fusion of vesicles on the unwalled droplets and the symbiosomes. So far, no symbiosome-specific exocyst tethering complex has been identified. The *M. truncatula* genome contains multiple genes (42), encoding the Exo70 subunit that is anchored on the target membrane and the other subunits of the octameric exocyst complex are duplicated, complicating the identification of the symbiosome-specific subunits even if some of them have a nodule-enhanced expression.

The secretory pathway uses filamentous actin as a scaffold for vesicle transport. Thus, the actin cytoskeleton in the symbiotic cells must be of primordial importance to guide vesicles of the exocytosis pathway towards the ensemble of symbiosis organelles in the cell. In agreement, the actin cytoskeleton architecture reorganizes during the differentiation of the symbiotic nodule cells (Gavrin et al., 2015; Zhang et al., 2019). Dense filamentous actin clusters around growing infection threads and infection droplets; then the actin filaments reorganizes around immature symbiosomes, forming a network around them, until when short actin filaments and actin dots form around the mature and radially aligned symbiosomes. Strikingly, the microtubular cytoskeleton closely parallels the changes in the actin cytoskeleton during infection thread growth, droplet formation, bacterial release, and symbiosome positioning (Timmers et al., 1998; Kitaeva et al., 2016). A key factor in the actin dynamics during the formation of the symbiotic cells is the ARP2/3 actin nucleation complex, which associates with maturing symbiosomes and might anchor the actin filaments to them. Silencing of the *ARP3* gene abolishes the differentiation of the symbiosomes (Gavrin et al., 2015). Another intriguing factor that might affect the actin cytoskeleton in the symbiotic nodule cells is queuosine, a modified nucleoside, produced by the bacteroids (Marchetti et al., 2013). Indeed, in a search for a suitable cellular experimental model system for bacterial uptake, it was found that *S. meliloti* can induce a strong actin cytoskeleton reorganization in human HeLa cells, and this response depends on bacterial queuosine production since *S. meliloti* mutants in the biosynthesis of this compound fail to induce the actin rearrangement. Interestingly, these mutants in queuosine synthesis form nitrogen-fixing nodules in which however the radial organization of the symbiosomes is lost, presumably because of an effect on the actin cytoskeleton of the host cell.

How the symbiosome organelle relates to lytic vacuoles is also partially understood thanks to the characterization of specific vacuolar marker proteins. Concomitantly with their gradual filling with symbiosomes, the volume of the vacuoles of the symbiotic nodule cells contracts temporarily and vacuoles are very small in young mature symbiotic cells but they become then larger again in older symbiotic nodule cells (Fig. 1) (Gavrin et al., 2014). This collapse of the vacuole permits the expansion of the symbiosomes and is most likely due to the suppression of the expression of the *VPS11* and *VPS39* genes encoding HOPS vacuolar tethering complex proteins. It could be of interest to check if this downregulation is mediated by the RSD repressor that downregulates VAMP72a. Moreover, the vacuoles of the infected nodule cells lose their functionality and are not acidic contrary to the vacuoles of uninfected cells. This defunctionalization of the vacuoles is further accompanied with retargeting of the aquaporin TIP1g from the tonoplast membrane to the symbiosome membrane. The relocalization

of TIP1g appears to be essential for the maturation of symbiosomes because they did not differentiate in RNAi knock-down lines of *TIP1g*. Thus taken together, the symbiotic nodule cells adjust the vacuole features as a mechanism that facilitates symbiosome formation. Later on in the existence of nodules, during senescence, lytic compartments appear in the symbiotic nodule cells. Accordingly, *VPS11* and *VPS39* are expressed again in these senescing symbiotic cells. Concomitantly, vacuolar t-SNAREs, the syntaxins SYP22 and VTI11, start to accumulate on the symbiosome membrane, indicating that the identity of the symbiosome compartment is changing towards a lytic compartment heralding the degeneration of the bacteroids they contain (Limpens et al., 2009).

The ensemble of these studies starts to draw a picture of the mechanisms that allow the symbiotic cells to sort out the symbiosomes within the endomembrane system of the cell and deliver them the resources they need for their growth and later on for nitrogen fixation. But clearly the picture is still out of focus and many questions remain open. To name just one: we do not know how the cargo proteins for the symbiosomes are sorted from the cargo to be delivered to the extracellular space or other cellular compartments. The symbiosomes do not have a cell wall, contrary to the extracellular space and thus the cell wall synthesis machinery must be targeted specifically to the plasma membrane. Moreover, the plasma and symbiosome membranes have a different complement of small metabolite transporters and a large number of secretory proteins are transported specifically to the symbiosomes. As argued above, VAMP721d/e vesicles might contain a different cargo than the VAMP721a vesicles. We have at present no clue how these proteins are properly sorted and addressed to the right compartment. In this respect, it is intriguing that the *dnf1* mutation only affects symbiosome maturation but not the formation of the unwalled droplets or the cell wall. Thus, the DNF1 signal peptidase complex might be specifically involved in the processing of the secretory proteins that are destined to the symbiosomes, but how this can happen is a mystery. Maybe the DNF1 SPC complex is only localized in the ER complexes that are in vicinity of the symbiosomes.

Among the cargo proteins destined for the symbiosomes are a large number of proteins that are uniquely expressed in nodules, including thioredoxin s proteins, GRPs, NCRs, SNARPs, calmodulin-like proteins, Enod8, Enod16, Enod20, DNF2, SymCRK, nodulin 25, MtN22, MtN11, lipase/lipoxygenases (nodule-specific PLAT domain proteins or NPDs) and basic blue-like protein. The corresponding genes are mostly very highly expressed, suggesting that a major activity of the symbiotic cells is the protein trafficking to symbiosomes, in agreement with the highly developed ER in these cells. However, the function of most of these proteins is not or only poorly known. One of the exceptions is the NCR peptides that control the terminal differentiation of the bacteroids in the symbiosomes.

Terminal bacteroid differentiation

After their release from infection threads, the rhizobia within the symbiosomes become functional bacteroids implying an alteration in their physiology to adapt for nitrogen fixation. This physiological switch is triggered by the low oxygen levels prevailing in the symbiotic nodule cells and is made possible by a complete rewiring of the bacterial transcription affecting a major fraction of the genes (Roux et al., 2014). Moreover, in *M. truncatula*, the physiological transformation of the bacteroids is also accompanied with the remarkable process of terminal differentiation. This bacterial differentiation process is irreversible or terminal, because the bacteroids are unable to resume growth when released from the nodules. The terminal bacteroids are characterized by a partially permeabilized cell membrane and by a very strong cell elongation leading to the formation of giant bacterial cells (Mergaert et al., 2006). Strikingly, these large bacteroids are polyploid, just like the symbiotic nodule cells in which they reside. Terminal differentiation of bacteroids is typical for particular legume clades, e.g. the IRLC to which *M. truncatula* belongs, or the Dalbergoid clade with *Aeschynomene* spp. However, it is not common to all legumes. In these other legumes, bacteroids are

in a reversible state, not displaying any of the distinctive features of terminally differentiated bacteroids. Their morphology, ploidy and membrane features are similar to the free-living rhizobia. This implies that the process of terminal differentiation is not an absolute requirement for obtaining functional nitrogen-fixing bacteroids, but it was proposed to improve the efficiency of the symbiosis (Mergaert, 2018). Phylogenetic analyses and a range of experiments that compare symbiotic performances of terminally differentiated bacteroids with less or non-differentiated ones confirm this hypothesis (Oono et al., 2010; Oono & Denison, 2010; Sen & Weaver, 1984; Kazmierczak et al., 2017; Lamouche et al., 2018; Lamouche et al., 2019). However, it is unclear at present why these large, partially permeabilized, polyploid bacteroids would be more performant than the undifferentiated ones.

The terminal differentiation is triggered by the above-mentioned family of antimicrobial peptides (AMPs) produced by the symbiotic nodule cells, called the nodule-specific cysteine-rich (NCR) peptides (Van de Velde et al., 2010). Many lines of evidence support this. NCR genes are only found in the so far analyzed legumes that have terminally differentiated bacteroids (Mergaert et al., 2003; Czernic et al., 2015). Within the IRLC clade, the extent of bacteroid differentiation varies depending on the plant and is correlated with the number of NCR genes in its genome, especially with the number of cationic ones (Montiel et al., 2017). In *M. truncatula* nodules, the NCR expression pattern overlaps with the differentiating and mature bacteroids (Guefrachi et al., 2014). Also in other IRLC legumes or in Dalbergioid legumes, tested NCR genes are only expressed in the bacteroid-containing nodule cells. Moreover, the NCR peptides accumulate in bacteroids. In agreement with this, the NCRs are indeed secretory peptides and their transport depends thus on the secretory pathway. In the *dnf1* mutant, the NCR peptides are retained in the ER and this correlates with the absence of bacteroid differentiation in this mutant as described above. In addition, mutants in some individual NCR genes are affected in bacteroid differentiation or in bacteroid persistence (see below). *In vitro* treatment of *S. meliloti* with synthetic NCRs resulted in bacteroid-like cells with permeabilized membranes, arrested division, cell elongation and DNA multiplication (Van de Velde et al., 2010). Most strikingly, the expression of NCR genes in *Lotus japonicus* plants, a legume that does not possess NCR genes and normally has reversible bacteroids, leads to bacteroids in the transgenic plants with features of terminal differentiation. And finally, rhizobium mutants that are affected in their response to NCR peptides are inept to differentiate into bacteroids (see below).

The NCR gene family is notable in several ways. In *M. truncatula*, over 600 NCR genes were identified in the genome, and in several other species of the IRLC clade, many hundreds of them are present as well (Mergaert et al., 2003; Montiel et al., 2017). Also in *Aeschynomene* species, several tens of NCR genes are expressed in nodules (Czernic et al., 2015; Gully et al., 2018). A transcriptome analysis in *M. truncatula*, using data of a very large number of experiments, found that the NCR genes are only expressed in nodules where they are moreover among the most highly expressed genes (Guefrachi et al., 2014). In addition, all NCR genes are specifically expressed in the symbiotic cells (except for two). The orthologous NCR genes in different *M. truncatula* accessions showed a high degree of nucleotide variation with signatures of diversifying selection pressures (Nallu et al., 2014), possibly reflecting an adaptation to the diversity of possible rhizobium symbionts. This could be one of the underlying reasons for the surprising large size of the NCR gene family. But other factors might have contributed as well. One of them is that the NCR genes are not functionally equivalent and are expressed in different phases of the symbiotic cell and bacteroid differentiation processes. Moreover, many of them are only expressed when bacteroids are already mature and fix nitrogen, suggesting that these “late” genes are involved in the maintenance of the bacteroid state rather than in the formation of it. Thus, it is expected that NCR peptides provoke many physiological changes in the bacteroids. Finally, it is possible

that deleterious side-effects on bacterial physiology of particular NCR peptides was compensated by additional peptides, also expanding the family (see below).

The NCR peptides have features shared with AMPs and at least some NCRs, and in particular the cationic ones, can *in vitro* kill or inhibit the growth of not only the rhizobium symbionts but also many other bacteria and even fungi (Farkas et al., 2018). Their major antibacterial activity results from their capacity to disturb the integrity of the inner and outer membranes of bacteria, leading to a loss of the membrane potential (Van de Velde et al., 2010). In addition, NCR peptides have intracellular targets, binding to ribosomes and inhibiting translation (Farkas et al., 2014). However, eliminating the endosymbionts is evidently not what happens in the symbiotic nodule cells and the bacteroids remain metabolically active for very long despite the deployment of this impressive NCR arsenal. How the antimicrobial activity of the NCR peptides relates to their *in vivo* action and induces terminal bacteroid differentiation is still unclear. Possibly, many factors within the symbiotic nodule cells and symbiosomes contribute to modulate the antimicrobial activity of the peptides to a much more subtle but complex effect on the endosymbiont physiology.

Indeed, endosymbiont adaptations contribute to the bacteria's ability to endure exposure to the NCR peptides and to colonize stably the symbiotic cells. Among them is the BacA peptide transporter in *S. meliloti* (known as BclA in the bradyrhizobia infecting the NCR-producing *Aeschynomene*). BacA-deficient rhizobia are hypersensitive to the killing activity of NCR peptides. Such mutants induce nodules and infect symbiotic cells similarly as in the wild-type. However, in NCR-producing symbiotic cells, the bacteria die as soon as they are released in the symbiotic cells and this death can be avoided by blocking NCR transport to the infecting rhizobia in the *M. truncatula dnf1* mutant (Haag et al., 2011; Guefrachi et al., 2015; Barrière et al., 2017). On the other hand, the BacA or BclA proteins, which are widespread and found in all rhizobia and even in other non-symbiotic bacteria, have no function in symbiosis in NCR-lacking legumes. BacA and BclA proteins are ABC peptide transporters with a broad spectrum of peptide import activity and they can promote the uptake of NCR peptides, suggesting that the transport of NCR peptides through BacA or BclA provides resistance. Other bacterial envelope components, including outer membrane proteins, LPS, EPS, peptidoglycan and hopanoids, are additional potential factors that contribute to NCR resistance and terminal bacteroid differentiation (Mergaert, 2018). For example, rhizobial EPS interacts with NCR peptides and is proposed to form a shield that can reduce the harmful interaction of NCR peptides with the bacterial membrane (Montiel et al., 2017; Arnold et al., 2018). Moreover, transcriptome and Tn-seq (transposon sequencing) analyses of NCR-treated cells and NCR-protein interaction studies identified a whole suite of additional candidate NCR-responsive functions in *S. meliloti* (Farkas et al., 2014; Penterman et al., 2014; Arnold et al., 2017). Together, these bacterial functions may contribute to alleviating the stress on the bacteroids induced by the NCR peptides. This suggests that the NCR peptides can act as determinants of compatibility between the host plant and the endosymbiont and that the peptides can function in the elimination of unadapted rhizobium strains. That this is truly part of the role of NCR peptides is illustrated by two NCR genes, named *NFS1* and *NFS2* that both exist in two allelic forms in different *M. truncatula* accessions (Wang et al., 2017; Yang et al., 2017). In the presence of a particular allele of *NFS1* or *NFS2*, some *S. meliloti* strains are not able to differentiate into functional bacteroids while other strains are not sensitive to these NCR alleles and differentiate normally. On the other hand, the sensitive strains are fully competent when infecting *M. truncatula* lines expressing other alleles of the *NFS1* and *NFS2* genes. This suggests that the *NFS1* and *NFS2* alleles leading to incompatible interactions encode peptides that directly or indirectly kill unadapted rhizobia. The sensitive strains produce a slightly different EPS than the compatible ones and transferring EPS biosynthesis genes from a compatible strain to the incompatible one overcomes the incompatibility (Simsek et al., 2007). These NCR alleles thus highlight how the bacterial adaptation to the NCR-profile of a host plant determines

the compatibility or incompatibility in a given symbiont-host genotype-genotype interaction. Some rhizobium species can even escape altogether the NCR-mediated reproductive inhibition and terminal bacteroid differentiation by producing the M16A family metallopeptidase HrrP capable of degrading NCR peptides (Price et al., 2015). HrrP expressing strains proliferate more abundantly within the nodules without fixing nitrogen for the host and have thus shifted from a cooperative to a parasitic behavior.

Well-adapted rhizobia resist the NCR challenge and establish chronically within the symbiotic nodule cells. They nevertheless do not emerge unaffected and rather undergo an irreversible physiological shift. The NCR peptides provoke a cell cycle transition, resulting from an inhibition of the bacterial cytokinesis, while at the same time DNA replication is maintained leading to polyploidy (Mergaert et al., 2006). The central cell cycle regulator CtrA has been proposed as a key target for the NCR peptides that promote this cell cycle switch (Penterman et al., 2014; Pini et al., 2015). Besides the cell cycle, many other cellular components were found to interact with NCR peptides, including the chaperone GroEL, which is also required for bacteroid differentiation, the metabolic enzyme pyruvate dehydrogenase, the F-ATP synthase and the nitrogenase enzyme complex (Farkas et al., 2014). These observations are compatible with the view that the NCR peptides interfere with a broad panel of bacterial functions. It is possible that the differentiation of the bacteroids by cell enlargement and polyploidization is a secondary effect induced by the AMP stress, while the primary effect is a manipulation of the metabolism of the endosymbiont in order to mold the bacterial metabolism for optimal nitrogen fixation. Two *M. truncatula* mutants, *dnf4* and *dnf7* (*defective in nitrogen fixation 4* and *7*), disrupted in the NCR211 and NCR169 encoding genes, respectively, form non-functional nodules in which bacteroids partially or even completely differentiate but they do not persist (Horváth et al., 2015; Kim et al., 2015). This was an unexpected finding, firstly because a high degree of redundancy was *a priori* expected in the very large NCR gene family. And secondly, because in the NCR169 and NCR211 mutant plants bacteroids differentiate, or at least partially differentiate, one has to conclude that the function of these peptides is not related to the cell cycle switch. Because well-adapted rhizobium strains die in the NCR169- and NCR211-deficient plants, the function of these peptides cannot be the elimination of unadapted bacteria either, as the restrictive *NFS1/2* alleles are doing. Thus, their role should be related to the interference with another cellular process. How to explain mechanistically the phenotype of these two mutants is presently not possible, but one hypothesis is that the NCR169 and NCR211 peptides have essential antagonistic or compensating activities to other NCR peptides that are toxic when not counteracted. Identification of suppressor mutations in either *M. truncatula* or *S. meliloti* that restores the symbiotic activity of the *dnf4* and *dnf7* mutants could be helpful to settle this question.

This picture of NCR peptides interfering with a broad range of bacterial functions is based on the study of a few peptides only, suggesting that the full scale of interactions of the NCR armamentarium is enormous. Moreover, this complexity could even be enhanced by mechanisms that modify the NCR peptides or their activity. The conserved cysteine residues of the NCRs likely form disulfide bridges during their passage through the oxidizing environment of the ER. The earlier mentioned Ero1- and PDI-encoding genes that are upregulated in the symbiotic nodule cells could contribute to the oxidation of NCRs in the ER. On the other hand, the symbiotic cells produce also a set of particular thioredoxins, *i.e.* proteins that catalyze the reduction of disulfide bridges in proteins to the free cysteines. These nodule-specific thioredoxins are coexpressed with the NCR peptides and are secretory proteins targeted to the symbiosomes and localized in the bacterial cytosol. They reduce NCR peptides *in vitro* and knocking down their expression by RNAi reduces significantly the level of terminal bacteroid differentiation while expressing the thioredoxin gene in the *S. meliloti* symbiont complements the bacteroid defect in the RNAi lines (Ribeiro et al., 2017). This complementation

supports the hypothesis that the thioredoxins reduce NCR peptides within the bacteroids and that the redox balance of NCR peptides is important. Accordingly, the redox state of the NCR peptides has *in vitro* a strong impact on the activity of the peptides. The antimicrobial activity, the activation or repression of gene expression, or the peptide stability towards the degradation by HrrP, were all affected by the NCR redox state (Haag et al., 2012; Shabab et al., 2016).

Many pathogenic bacteria inject “effector” proteins into host cells as part of their pathogenicity strategy. These effectors suppress host immune responses and subvert intracellular processes, thereby promoting bacterial colonization of the host. Pathogens usually inject a large diversity of effectors, up to over a hundred different proteins. A picture that emerges from the above is that the NCR peptides are comparable to these effectors but acting in the opposite direction: they are produced by the host and modulate bacterial processes. The NCR effectors might be a strategy to limit the possibilities of the bacteroids to act against the interests of the host. On the other hand, the peptides should not overly act and kill the symbionts while the latter have possibilities for counteracting the effectors. Thus, a subtle balance between the manipulation of the bacterial physiology by the NCR effectors and the ability of the rhizobia to resist them is required for a functional symbiotic association. An imbalance, as seen in bacterial and plant mutants or natural variants, results in a breakdown of the symbiosis.

The large number of bacteroids in the symbiotic cells implies the massive presence of microbial molecules that can potentially act as pathogen-associated molecular patterns (PAMPs) and induce a strong PAMP Triggered Immunity (PTI). To prevent such a response, which would be destructive to the symbiosis, the symbiotic cells of *M. truncatula* nodules activate a specific machinery that enables the long-term maintenance of the rhizobia without mounting a PTI response (Fig. 3) (Bourcy et al., 2013; Berrabah et al., 2014; Wang et al., 2016; Domonkos et al., 2017). In the absence of the symbiotic cysteine-rich receptor-like kinase SymCRK or the phospholipase C-like DNF2, both probably located in the symbiosomes, or the ER located small protein NAD1 (Nodules with Activated Defense 1), a strong immune response is induced, observable by the induction of marker genes of immunity, nodule necrosis and a complete halting of the symbiosis. The PTI response was reported to be dependent on ethylene signaling in the *symcrk* and *dnf2* (*defective in nitrogen fixation 2*) mutant nodules (Berrabah et al., 2018) and on a signaling cascade involving respiratory burst oxidase homologs (Rboh) and a calcium-dependent protein kinase (CDPK) in the *nad1* nodules (Yu et al., 2018). Both pathways are known regulators of PTI. At present, it is still unclear how the three nodule suppressors of immunity interfere with these pathways. However, it is unlikely that they act at the transcriptional level, given their localization in the ER or the symbiosomes. It is also unclear if other immunity pathways are suppressed and in how far the three suppressors act on overlapping or separate pathways. However, by combining the immunity suppressor mutants with the above-described *exoY*, *ipd3*, *lin*, *bacA* and *dnf1* plant and bacterial mutants affected in different stages of infection or bacteroid differentiation, it became clear that the suppressors, which are only expressed in the symbiotic nodule cells, only act after release of rhizobia from infection threads. Moreover, they act at different steps thereafter, at the bacterial uptake step for NAD1 and DNF2, and at the formation of mature bacteroids for SymCRK (Berrabah et al., 2015; Domonkos et al., 2017). This suggests that depending on the stage of bacteroid differentiation, different PTI responses are activated and that there is a corresponding specialization of the three immunity repressors. Furthermore, the immune suppression by NAD1, DNF2 and SymCRK was proposed to be specific for the terminally differentiated bacteroids since equivalent mutants in *L. japonicus*, which has reversible bacteroids, have no phenotype whatsoever (Yu et al., 2019). Thus, the challenge with the NCR antimicrobial peptides possibly provokes the release of PAMPs, requiring the specific suppression of PTI.

Gene expression and regulation in the symbiotic cells

Genome-wide gene expression studies have been performed extensively in *M. truncatula*, revealing that nodule formation is accompanied by the differential expression of several thousand genes. The analysis of the expression pattern of these nodule enhanced genes showed that a large proportion of them is extremely specific and only expressed in nodules (Guefrachi et al., 2014). The entire *NCR* gene family for instance is absolutely nodule specific as mentioned earlier. In addition, transcriptome studies providing spatial resolution at the cell or nodule-zone level, by using nodulation mutants or by combining laser-capture microdissection with RNA-seq, demonstrated that the large majority of the nodule-enhanced or nodule-specific genes is expressed in the differentiating or mature symbiotic cells (Maunoury et al., 2010, Roux et al., 2014).

Stimulation of the CSSP by Nod factors results in the activation of a well-characterized TF network that triggers the infection process and nodule organogenesis. However, how this TF network further connects to the downstream massive reprogramming in the symbiotic cells still has to be discovered. This regulation most likely involves a presently unknown downstream TF network. An important challenge for the future is to identify these TFs and their connection to the known upstream regulatory pathways.

Moreover, the strict expression of the nodule-specific genes suggests that regulation at the chromatin-level is important as well. Indeed, genes with high tissue- or condition-specific expression are often actively silenced by epigenetic mechanisms that are reversed when expression is required, giving TFs access to their target genes. Indications for such a key role of chromatin-based regulation in the massive gene activation during symbiotic cell differentiation were obtained by analyzing the chromatin features of the nodule-specific genes. Because of the coupling of differentiation to endoreduplication, symbiotic nodule cells multiply their genome from a 2C and 4C DNA content in meristem cells, over 8C and 16C in differentiating cells, till 32C and 64C in mature symbiotic cells (Vinardell et al., 2003). Using purified nodule nuclei of these various ploidy levels, a correlation was found between the ploidy level of the cells and the sequential activation of nodule specific genes, suggesting that the endoreduplication is implicated in the gene expression control (Nagyimihály et al., 2017). Expression of the nodule-specific genes in the different ploidy-types further correlated with opening of the chromatin as well as with reduced levels of the chromatin mark H3K27me3 (Nagyimihály et al., 2017). H3K27me3 (trimethylation of lysine 27 in histone H3) is a repressive chromatin mark, introduced by the Polycomb silencing complex. In another study, it was noticed that genes that are specifically expressed in the differentiating and mature symbiotic cells in *M. truncatula* often display local duplications and are clustered together with other types of nodule-specific genes (Pecrix et al., 2018). These genome clusters of nodule-specific genes were called symbiosis-related islands (SRIs). Almost 300 SRIs were identified and they contain on average more than seven nodule-enhanced genes of more than five distinct gene families. The SRIs showed reduced H3K27me3 levels in nodules compared to roots. Thus, the activation of nodule-specific genes could be made possible by opening of the chromatin and release of H3K27me3-mediated repression during the differentiation of the symbiotic cells.

Promoter DNA methylation is another level of epigenetic gene silencing. It was found, by comparing the DNA methylation levels either in whole roots versus nodules or in nodule nuclei of different ploidy level, that many of the symbiotic nodule cell-specific genes, including genes encoding the NCRs, become demethylated in correlation with the activation of their expression (Satgé et al., 2016; Nagyimihály et al., 2017). Remarkably, the SRIs displayed such a differential methylation pattern in roots versus nodules and the borders of the SRIs sharply delineated the limits of the differentially methylated regions while the SRI-flanking regions showed very low and non-differential methylation (Pecrix et al., 2018). This DNA demethylation of the nodule-specific genes is in large part mediated by

the DNA demethylase DEMETER that is transcriptionally activated in the differentiating symbiotic cells (Satgé et al., 2016). Furthermore, the SRIs contain numerous expressed long non-coding RNAs, potentially also involved in the regulation of the SRI genes (Pecrix et al., 2018).

Together, these findings suggest thus that nodule-specific genes and the SRIs are silenced by epigenetic means, by DNA methylation and by histone modifications, and that endoreduplication in the differentiating symbiotic cells and the expression of long non-coding RNAs correlates with their epigenetic reprogramming in such a way that they become accessible and competent for transcription. However, the current data only establishes correlations between gene expression on the one hand and chromatin modifications or endoreduplication on the other hand. Clearly, a challenge for future studies will be to establish causal relations between these processes and to identify the underlying molecular mechanisms.

Conclusion: the integrated picture

The formation of the symbiotic nodule cells recruits existing and widely used plant processes. Indeed, meristems provide the cells for all plant tissues; endoreduplication is found in many plant cell types and depends on the same molecular mechanism in nodules and elsewhere; the tip growth of infection threads is shared with root hairs or pollen tubes; endocytotic and exocytotic vesicle trafficking and actin cytoskeleton rearrangements are common in all plant cells; antimicrobial peptides are part of the innate immune system of plants; and the nitrogen and carbon metabolism of the symbiotic cells relies on enzymes participating in the central metabolism of plants. However, these common processes are integrated with completely new features not seen anywhere else in plant biology. The organization of the nodule meristem providing lateral vascular bundles and a central endoreduplicating tissue is unlike any other meristem. Infection threads are trans-cellular and subcellular structures that use cell cycle and polar growth-related processes but they are not autonomous cells like root hairs or pollen tubes. Endocytosis of whole bacteria is not known elsewhere in plants. The symbiosomes may resemble phagocytized pathogens in animals but in plants, such new organelle-like structures are unique. A heavy load of bacteria inside plant tissues as in the nodules would normally lead to an immune response or a collapse of the plant tissue and a unique deployment of immune effectors and immune suppression is therefore required. The metabolic integration of the bacterial endosymbionts needs the management of specific metabolic fluxes. Finally, the whole process involves the expression of a very large set of genes that are only expressed in the symbiotic cells. These genes arose from gene duplications combined with modifications leading to neofunctionalization (for example the oxygen transporting leghemoglobins, derived by replacement of a few amino acids from non-oxygen-transporting hemoglobins present in all plants; Sturms et al., 2010). Others arose from duplications whereby one paralog obtained a new, nodule-specific expression profile but without acquiring a new protein function (for example the secretory pathway genes or small metabolite transporters and metabolic enzyme genes). And still other symbiotic cell-specific genes were formed *de novo* and have no homologs (so-called orphan genes such as the *NCRs* as well as many others).

The net result of this tinkering with old and new parts is this particular cell type packed with nitrogen-fixing organelles that is very efficient for nitrogen and carbon exchange and that can produce sufficient reduced nitrogen to meet all the needs of the plant. All nitrogen-fixing nodule-forming plants, including legumes and actinorhizal plants, have some form of symbiotic nodule cells similar to the ones of *M. truncatula* described here, even if they might differ in many details (Sprent et al., 2017; Pawlowski & Demchenko, 2012). In the light of the ongoing discussion on the evolution of the nodule-forming symbiosis in legumes and actinorhizal plants (van Velzen et al., 2019; Parniske, 2018), a fascinating question to think about is if the symbiotic nodule cell is ancestral in this symbiosis or if it appeared by convergent evolution in the different nodule-forming lineages. One can also imagine that, in case they

are indeed ancestral, they have first existed in root cortical cells independently of organogenesis in early evolution of the symbiosis, in a similar form as the arbuscules in the mycorrhizal symbiosis (Lanfranco et al., 2016). The nodule formation itself has then subsequently evolved by recruitment of cell divisions and the gradual appearance of additional cell types. Part of this evolutionary process could have taken place in the common ancestor of nodulating plants and part of it could have appeared in parallel in the different lineages.

Finally, it is interesting to note that cells with many features analogous to symbiotic nodule cells are found in insects that maintain nutritional endosymbionts in specific symbiotic cells called bacteriocytes (Baumann, 2005). These bacteria synthesize amino acids or vitamins for their host instead of the reduced nitrogen produced by the rhizobial symbionts of legumes. At least in the case of symbiotic nodule cells versus bacteriocytes, evolution was convergent. It has come up multiple times with a similar solution for the stable maintenance of a very large number of bacteria within a cell and for the establishment of an efficient metabolic interaction between eukaryotic cells and bacterial endosymbionts.

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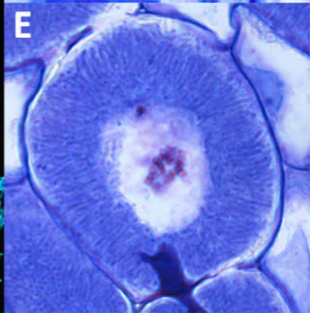
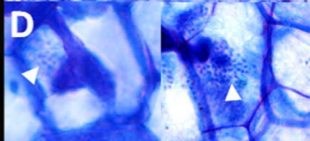
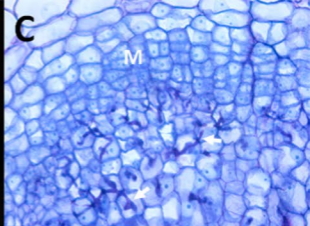
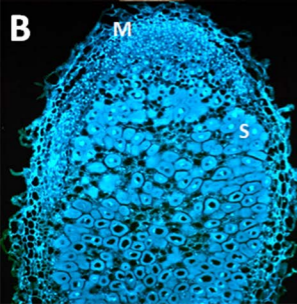
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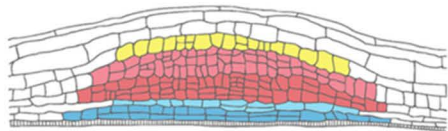
Figure legends

Figure 1. *Medicago truncatula* root nodules. **A.** Nodules on a root of a *M. truncatula* plant. **B.** A nodule section stained with DAPI showing small dividing cells with small nuclei in the meristem (M), and symbiotic cells (S) with large nuclei in the central zone of the nodule. **C.** Close-up of a nodule meristem and the underlying differentiating symbiotic cells that are crossed by infection threads (arrows). **D.** Differentiating symbiotic cells with freshly released rhizobia (arrowheads). **E.** A symbiotic nodule cell filled with radially aligned bacteroids.

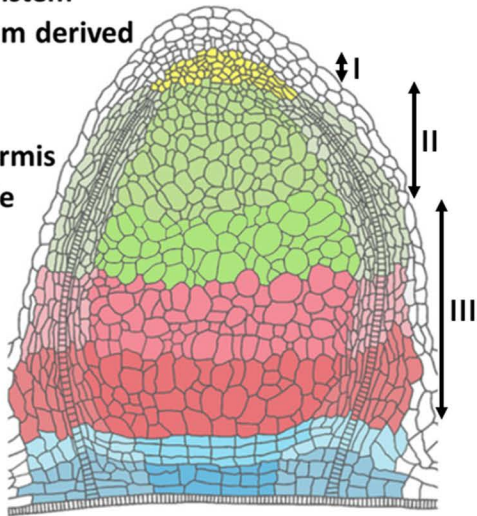
Figure 2. Nodule organogenesis and differentiation of symbiotic cells. **A.** Top: Primordium stage in which anticlinal and periclinal divisions occurred in the C3, C4, C5 cell layers and in the endodermis and pericycle, resulting in the first new cell layers and a visible outgrowth of the incipient organ. Bottom: Mature nodule with the six basal uninfected cell layers derived from the pericycle and endodermis, the eight most basal infected cell layers derived from the C4 and C5 root cortical layers and the remaining infected cells derived from the meristem. The color key shows the cell fate map of the primordium and nodule tissues. **B.** Differentiation of symbiotic cells at the margin of the nodule meristem. Meristem cells that have replicated their genome (passed through the S phase of the cell cycle) have two possible fates: either they complete the cell cycle, go through mitosis (M phase) and divide to generate two new meristem cells (1) or they prepare for infection and differentiation into a symbiotic nodule cell (2). Entry into the differentiation pathway is triggered by the penetration of an infection thread and the endocytotic release of rhizobia, a process that is regulated by Nod factor production by the rhizobia in the infection threads and by the plant common symbiosis signaling pathway (3). The differentiation requires a cell cycle switch driven by the Ccs52A protein (4). The cell cycle switch produces polyploid and large symbiotic cells. Panel A is republished with permission of "The Company of Biologists Ltd", from Xiao et al., 2014.

Figure 3. Differentiation of bacteroids in symbiotic nodule cells. An infection thread releases rhizobia from unwalled infection droplets by endocytosis. The trafficking of secretory pathway vesicles to infection droplets and symbiosomes delivers nodule-specific cargo proteins (green, orange, and yellow globules). For example, cell wall degrading enzymes (green globules) are targeted to the droplets. The NCR bacteroid differentiation factors (orange globules) are targeted to the symbiosomes. Immune suppressor proteins (yellow) are targeted to the ER or the symbiosomes containing differentiating bacteroids. The actin cytoskeleton (green filaments) guide the secretory vesicles to the target organelles. The symbiotic cells produce also nodule specific soluble proteins (purple globules), for example leghemoglobin and enzymes for ammonium assimilation or carbon delivery to the symbionts. The inset shows a detailed view of the identified SNARE and Rab proteins involved in vesicle targeting to symbiosomes. The synaptotagmins bind calcium (red) to control the membrane fusion mediated by the SNARE proteins. The exocyst tethering complex is depicted in green.



A

- C3/Meristem
- Meristem derived
- C4
- C5
- Endodermis
- Pericycle

**B**