Chapter

# Development and Resource Exchange Processes in Root Symbioses of Legumes

*Debatosh Das, Salina Tripathi, Prema Mutyala, Dhruv Aditya Srivastava and Heike Bücking*

## Abstract

Plants are associated with complex microbiomes, and many of the microorganisms that reside on plant surfaces (epiphytes) or within plant tissues (endophytes) are beneficial for the host plant and improve plant growth or stress resistance by a variety of plant growth-promoting capabilities. The plant microbiome could serve as a tool box to design synthetic microbiomes to enhance plant growth and crop resiliency under stress or to integrate benefits of plant microbiomes as important traits into plant breeding programs. For legumes, the most important members of the plant microbiome are nitrogen (N)-fixing rhizobia and arbuscular mycorrhizal (AM) fungi. Legumes harbor rhizobia in specialized root nodules, in which the bacteria fix gaseous N from the atmosphere and transfer plant available forms of N to host. AM fungi play a key role for the uptake of nutrients such as phosphate and nitrogen and improve the resistance of plants against abiotic (e.g. drought, salinity, and heavy metals) and biotic (herbivores and pathogens) stresses. Both partners compete with these benefits for photosynthetically fixed carbon from the host. In this review, we will summarize our current understanding of these interactions and will also focus on cooperative or competitive interactions between these two root symbionts in tripartite interactions.

**Keywords:** arbuscular mycorrhizal symbiosis, biological nitrogen fixation, mutualism, nutrient uptake, plant biotic interaction, rhizobia

## 1. Introduction

Plants are sessile organisms, and to compensate for their lack in mobility, plants evolved specialized mechanisms that allowed them to adapt to changing environments and to a variety of abiotic and biotic stresses. Arguably, the most important adaptation to stress was the development of beneficial plant microbe interactions. Due to recent developments in sequencing technologies, we have a better understanding of the concept that plants are meta-organisms, whose phenotype, particularly under stress, is not only shaped by plant traits but also by their associated microbiomes. The plant microbiome represents "the second plant genome" and consists of 10 times more genes than typical plant genomes and is an unexplored resource for a wide

range of potentially plant growth-promoting capabilities [1]. A better understanding of beneficial plant microbe interactions could be a key to the development of microbial fertilizers or microbial pesticides, as well as new biotechnological tools that increase the nutrient efficiency and stress tolerance of crops in environments that are increasingly affected by climate change and other stresses.

The most important root symbioses for legumes are interactions with nitrogen-fixing rhizobia and arbuscular mycorrhizal (AM) fungi. Legumes harbor rhizobia in specialized root organs or nodules, and their biological nitrogen (N) fixation can contribute with up to 77% to the total N nutrition of crop legumes [2]. AM fungi colonize the majority of land plants and transfer nutrients such as phosphate (P), N, and potassium (K) to their host and improve the host plant's resistance against abiotic (drought, salinity, and heavy metals) and biotic (herbivores and root pathogens) stresses [3]. Arbuscule-like structures found in the cells of early land plant fossils suggests that the AM symbiosis played a key role during the evolution of land plants, and the AM symbiosis is therefore also called "the mother of all root endosymbioses" [4, 5]. We will focus here on the symbiosis between legumes, rhizobia, and AM fungi and will summarize our current knowledge about the development and nutritional benefits of these interactions and discuss knowledge gaps that still limit their application potential.

## 2. Beneficial root symbioses of legumes

### 2.1 Arbuscular mycorrhizal symbiosis

The arbuscular mycorrhizal (AM) symbiosis is a mutualistic interaction between approximately 70% of all known land plant species and fungi of the phylum *Glomeromycota* [6]. AM fungi are ubiquitous in soils, and the extraradical mycelium (ERM) of the fungus can account for up to 50% of the microbial biomass in soils [7]. The ERM of the AM fungus can explore a larger soil volume, mine for nutrients beyond any root depletion zone, and transfer these nutrients to the intraradical mycelium (IRM) in the host root, where these nutrients are exchanged against carbohydrates and lipids from the host. The AM fungus provides the host plant with soil nutrients, such as P, N, K, and sulfur (S), but also trace elements, such as copper and zinc. In addition, AM fungi provide nonnutritional benefits to their host plant and improve the resistance of plants against various abiotic (drought, salinity, and heavy metals) and biotic (pathogens and herbivores) stresses [3]. Due to the key role that AM fungi play for the survival and fitness of plants, they have also been described as "ecosystem engineers" of plant communities [8].

Roots that are colonized with AM fungi have two pathways for nutrient uptake: the plant uptake pathway (PP) and the mycorrhizal uptake pathway (MP). The PP involves the uptake of nutrients from the soil via high- or low-affinity uptake transporters in the epidermis or root hairs. However, nutrients such as P are relatively immobile in the soil, and the efficiency of the PP is often limited by the development of depletion zones around the roots. The MP, on the other hand, is characterized by the uptake of nutrients from the soil via high-affinity nutrient transporters in the ERM, followed by the translocation of nutrients from the ERM to the IRM in the root cortex, and the uptake of nutrients from the mycorrhizal interface through AM-inducible plant uptake transporters. In AM roots, the MP represents often the dominant pathway for plant nutrient uptake [9, 10].

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AM fungi and their plant partners form a complex network of many-to-many interactions; each plant host is colonized by communities of AM fungi, and AM fungi colonize multiple host plants and connect plants via common mycorrhizal networks (CMNs). CMNs are involved in the long-distance transport of nutrients, water, stress chemicals, and allelochemicals and allow plants to "communicate" with other plants of the same or of different species that share the same CMN [11–13]. Many-to-many interactions allow both partners in the AM symbiosis to choose among multiple trading partners and force both partners to compete with other partners for nutrient or carbon resources.

The colonization of the root by AM fungi is initiated through a bidirectional exchange of signals. Before the AM symbiosis is established, plants release the carotenoid-derived plant hormone strigolactone, which activates fungal metabolism and stimulates hyphal branching during the pre-symbiotic growth phase (**Figure 1**) [14–16]. In addition to their role in plant partner recognition in parasitic and beneficial plant microbe interactions, strigolactones play a key role for the adaptation of plants against a variety of abiotic stresses and actively participate within regulatory networks of plant stress adaptation regulated by phytohormones. Abiotic stresses that have been shown to affect strigolactone production and/or the expression of genes involved in strigolactone biosynthesis include P and N deficiency, heat, UV, wounding, salinity, and drought [17]. For example, as part of the P starvation signal activated by PHR proteins in plants, strigolactone biosynthesis increases [18]. In response to strigolactones, AM fungi secrete Myc factors that are composed of lipo-chitooligosaccharides (LCOs) and chitin oligomers and are likely recognized by LysM-receptor-like kinases of the plant (RLKs) and prepare the root for colonization.

The developmental events in the establishment of the AM symbiosis include epidermal penetration, restructuring of the underlying epidermal and cortical cells, the assembly of a pre-penetration apparatus (PPA), intraradical colonization by hyphal elongation, and the development of arbuscules in inner cortical cells (**Figure 1**). After fungal hyphae have established contact with the host root, the AM fungus forms an appressorium or hyphopodium, a specialized cell or adhesion structure on the root surface. After the recognition of AM fungal appressoria, root epidermal cells develop

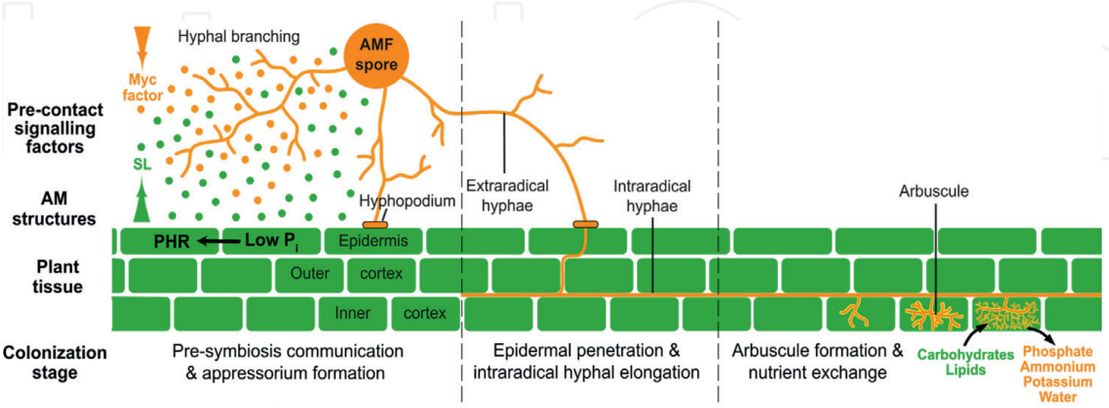


**Figure 1.**
*Pre-symbiotic signaling and intraradical development in the AM symbiosis. Under phosphate starvation, PHR-induced strigolactones (SL) are released by roots and activate the AM fungus, which in response releases Myc factors that are perceived by LysM-receptor-like kinases of the plant. The fungus forms an appressorium on the root surface after successful plant recognition. When AM fungal appressoria are detected, epidermal cells produce a pre-penetration apparatus (PPA) that guides AM fungal hyphae through the epidermis into the root cortex. Characteristic for the AM colonization is the development of arbuscules, highly branched structures in cortical cells, which play a critical role for the bidirectional exchange of nutrients across the mycorrhizal interface.*

a PPA, which directs AM hyphae through the epidermis into the root cortex and controls the intracellular path of hyphal penetration [19]. Arbuscules are highly branched structures that act as sites of nutrient exchange between the fungal and plant partner. Concurrent with these morphological events, a signaling cascade involving receptor kinases, nucleoproteins, ion channels, and a transcriptional complex takes place to accommodate the fungus inside roots (see 2.3 below).

### 2.2 The rhizobium-legume symbiosis

Legumes are characterized by their ability to establish symbiotic interactions with diazotrophic soil bacteria (known as rhizobia). Rhizobia can fix gaseous $N_2$ and convert it into plant-available forms of N in specialized root structures or nodules. Critical for the establishment of the symbiosis between legumes and rhizobia is a two-way recognition process between both partners. A key component of this recognition process is the secretion of flavonoid compounds by plant roots, which are recognized through bacterial NodD receptors and induce the biosynthesis of bacterial lipo-chitooligosaccharides or Nod-factors. The production of flavonoids and the expression of chalcone synthase (the first committed enzyme of flavonoid biosynthesis) and isoflavone reductase (conversion of flavanones to isoflavones) is triggered by N deficiency of the plant [20]. NodD belongs to the family of LysR-type transcriptional regulators that mediate the expression of nodulation (*Nod*) genes in rhizobia. Common in all bacteria that nodulate legumes are the *Nod* genes *nodABC,* which are all responsible for Nod factor biosynthesis [21]. Nod factors released by rhizobia are composed of lipo-chitooligosaccharides and are perceived by LysM-domain receptor-like kinases, such as NFR1 and NFR5 in *Lotus japonicus* [22, 23]. The perception and recognition of Nod factors by the host plant triggers a series of plant responses involved in nodule development and infection.

Unlike the AM symbiosis, the rhizobium-legume (RL) symbiosis is highly specific, and each rhizobial strain establishes a symbiosis with only a limited number of host plants and vice versa [24]. The ability of rhizobial species to recognize and interpret specific flavonoid signals produced by compatible host plants through NodD is in part responsible for the host specificity in RL interactions [25]. There is also a high level of specificity at the N-fixing stage in the RL symbiosis. Bacterial strains can form N-fixing root nodules on one plant genotype ($Nod^+/Fix^+$) and nodulate other plant genotypes, but the formed nodules of this plant are unable to fix N ($Nod^+/Fix^-$). This specificity is caused by the nodule-specific cysteine-rich (NCR) peptides NFS1 and NFS2 of the plant, which induce bacterial cell death, and early nodule senescence dependent on the rhizobial strain and the genetic background of the host. It has been suggested that NCR peptides possess prosymbiotic and antisymbiotic properties and that they play a role in fine-tuning the activity of rhizobia for optimum symbiotic performance [26].

Parallel to the change in root morphology, which includes cortical cell division and differentiation into nodule primordia, rhizobia are directed into the cortex. Rhizobia can enter roots through nod-dependent infection pockets formed during root curling via tubular infection threads (ITs) or cracks in the root surface. In addition, rhizobia can also gain entry via intercellular spaces through a nod-factor-independent process [27]. Necessary for most infections in legumes, however, is root hair curling, which involves root hairs that are tightly bending and entrap bacteria in a “shepherds crook” structure [28]. This deformation of root hairs is typically only induced by the release of Nod factors from bacteria closely attached to root hair tips. In some legumes,

the Nod factor (NodRm-1 in *Medicago sativa*) alone can elicit cortical cell division and root hair deformation [29]. In other cases, Nod factors induce oscillations in intracellular calcium levels, which later activate calcium- and calmodulin-dependent kinase (CCaMK), a symbiotic signaling pathway that is important in the activation of transcription factors critical for nodule organogenesis [30].

In legumes, two types of root nodules can be distinguished. Indeterminate nodules develop after Nod factor perception as a result of periclinal cell divisions in the pericycle followed by inner cortical cell proliferation. Indeterminate nodules are characterized by a persistent meristem and the development of distinct zones within the root nodule: meristem, infection zone, interzone, fixation zone, and senescence zone. By contrast, determinate nodules have a defined lifespan and lose their central meristem. Determinate nodules develop by cell divisions in the outer root cortex, but the cells lose their meristematic activity when the nodule matures [31]. The bacteria are taken up by a process similar to endocytosis of the plasma membrane where bacteria proliferate into N-fixing bacteroids and form a symbiosome, an organelle-like structure consisting of 1–10 bacteroids enclosed by a plant-derived peribacteroid membrane or symbiosome membrane (SYM) and the symbiosome space that is located between the bacteroids and the SYM [32].

### 2.3 The common symbiosis signaling pathway

AM or RL symbiosis play a critical role for the nutrient supply of their host, and low $P_i$ or N supply conditions have been shown to stimulate the release of strigolactones or flavonoids by host plants to recruit AM fungi or rhizobia. AM fungi and rhizobia respond to these signals with the release of Myc or Nod factors, respectively, which are composed of sulfated or non-sulfated lipo-chitooligosaccharides [33]. The perception and interpretation of Myc or Nod factors by the host plant plays a critical role in the establishment of both root symbioses. Compared to the AM symbiosis, which evolved around 450 to 480 million years ago, the mutualistic RL symbiosis is much younger and evolved approximately 58 million years ago [34]. The similarities in the signaling pathways of the AM and RL symbiosis has led to the assumption that the RL symbiosis evolved via the adoption of a signaling pathway that had previously been established for the successful colonization of plants by AM fungi. Similarly, the same signaling pathway can also be found in ectomycorrhizal associations that evolved also much later than the AM symbiosis [35, 36]. The common symbiotic signaling pathway (CSSP) is a conserved molecular signaling pathway that plays a key role in the establishment of the AM and RL symbiosis and acts downstream of Myc and Nod factor perception and upstream of the activation of processes required for the root colonization by specific root symbionts. Mutations in the CSSP prevent both fungal and bacterial entry into the host root [37].

The first component of the CSSP are heteromers of plasma membrane-localized lysin motif-type receptor-like kinases, such as the Nod factor receptors NFR1/NFR5 in *Lotus*, or LYK3/NFP in *Medicago* [38, 39], or the Myc factor receptor MYR1/LYK2 and CERK1 in rice [40]. NFR1 and NFR5 can both bind to Nod factors, but only NFR1 has kinase activity and can phosphorylate NFR5 and initiate the signal transduction cascade [38]. Kinase activities are essential for their function in rhizobial symbiosis. Some receptor kinases also seem to play a role in the recognition of chitin oligosaccharides in the cell walls of pathogenic fungi. For example, the fact that *CERK1* knockout mutants in rice are not only impaired in AM development but also in chitin-triggered defense responses indicates that CERK1 plays not only a role in mutualistic but also in parasitic interaction recognition [41].

Downstream of Myc or Nod factor perception, the symbiosis receptor-like kinase SYMRK is indispensable for the development of the AM or rhizobial symbiosis. In *Lotus japonicus*, SYMRK forms a receptor complex with NFR5 [42], and it was shown that SYMRK is able to suppress BAK1 kinase activity (BRASSINOSTEROID INSENSITIVE 1-Associated receptor Kinase 1). BAK1 is a positive regulator of plant innate immunity, and its inhibition by SYMRK could indicate that rhizobia actively suppress the host's immune response [43]. Only minutes after Nod factor perception, an activated oscillation in cytoplasmic $Ca^{2+}$ concentrations in the perinuclear region can be detected. In *Lotus japonicus*, two ion channels (CASTOR and POLLUX) [44, 45] and three nuclear pore proteins (NUP85, NUP133, and SEH1) [46, 47] act in a signaling cascade to generate this epidermal calcium spiking. This calcium spiking leads to a derepression of a nuclear-localized calcium calmodulin-dependent kinase (CCaMK) [48]. Subsequently, CCaMK (DMI3) phosphorylates the transcription factor CYCLOPS (IPD3) [49]. The result is a symbiont-specific transcriptional reprogramming, which is necessary to support the colonization of the root by AM fungi or start nodule organogenesis (**Figure 2**).

After the CSSP, specific signaling pathways regulate the development of the AM or RL symbiosis. In the AM symbiosis, molecular signaling coincides with the initiation of a PPA in plant cells to accommodate the invading fungus [50]. Subsequent fungal infection involves hyphal elongation and cortical root colonization and arbuscule formation. Arbuscule formation is regulated by a complex consisting

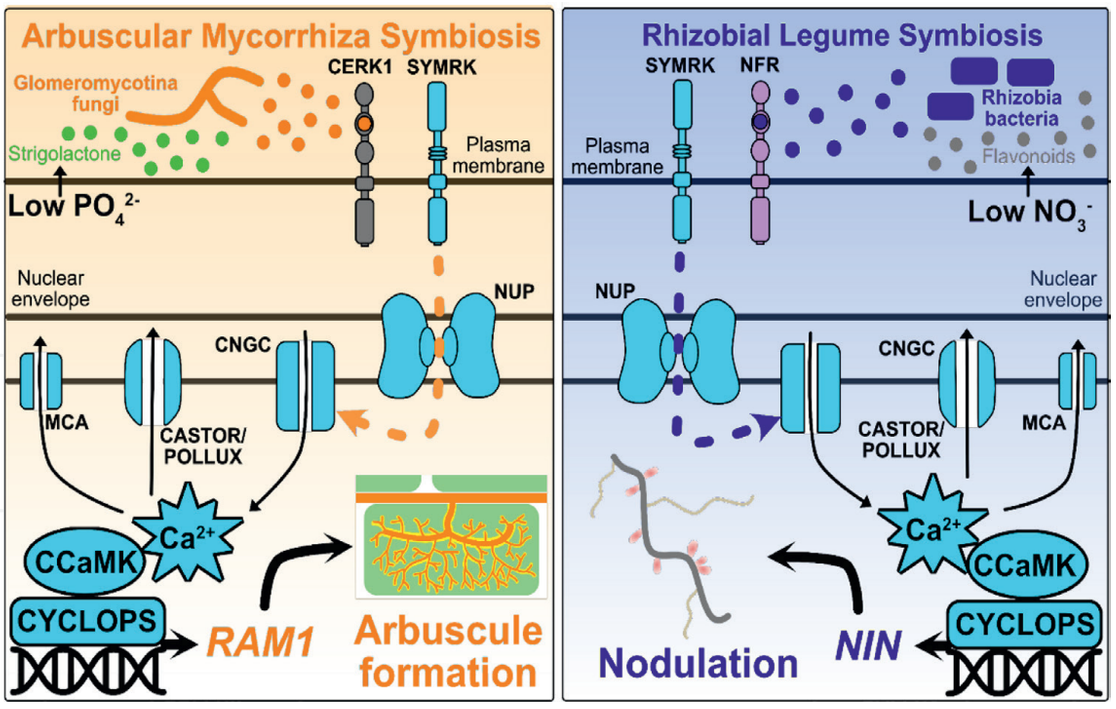


**Figure 2.**
*Common Symbiosis Signaling pathway (CSSP). In pre-symbiotic stages, SL and flavonoids are released by plants to attract AM fungi and rhizobia. In return, both symbionts release Myc or Nod factors that are perceived by CERK1 or NFR in the plasma membrane. Signals generated at SYMRK pass through the nucleopore protein complex (NUP) and lead to the activation of ion channels and ATPases (CNGC, CASTOR/POLLUX, and MCA) and calcium spikes in the nucleus. Calcium spiking activates the kinase CCaMK, which phosphorylates and activates the transcription factor CYCLOPS. The CCaMK-CYCLOPS complex regulates AM or RL symbiosis-specific transcription through the RAM1 or NIN transcription factor, respectively. This leads to intraradical colonization and the development of arbuscules in the AM symbiosis and infection thread formation and primordia development in the RL symbiosis.*

of CCaMK, CYCLOPS, and DELLA proteins, which induce the essential GRAS gene *RAM1* and are accompanied by an induction of AM transporter genes such as *PT4* and *AMT2;3* [51]. In rice, DELLA interacts with another GRAS protein, DIP1 (DELLA Interacting Protein 1), which in turn interacts with RAM1, and affects AM-induced gene expression [52].

By contrast, in the RL symbiosis, several nuclear-associated transcriptional regulators are essential for the expression of Nod-factor-induced genes and the initiation of nodulation, including nodule inception (NIN) [53], an ERF family protein (ERN) [54], and two GRAS family proteins, nodulation signaling pathway 1 (NSP1) and NSP2 [55, 56]. DELLA proteins promote nodule development and infection thread formation during root nodule symbiosis by promoting CCaMK-IPD3/CYCLOPS complex formation and increasing IPD3/CYCLOPS phosphorylation. DELLAs can also form a protein complex with NSP2 and NSP1 and bridge a protein complex containing IPD3/CYCLOPS and NSP2. It has been suggested that the combination of transcription factors such as NSP2 and NSP1 and CCaMK-IDP3 may act in tandem to control the expression of early nodulin genes [57]. First, the phosphorylated form of CYCLOPS (IPD3 in *Medicago truncatula*) binds to the NIN promoter and induces nodulation even in the absence of rhizobia [49], followed by the NSP1-NSP2 hetero complex, which binds to the promoters of the Nod-factor-inducible genes *ENOD11*, *ERN1*, and NIN [58]. Interestingly, NSPs are not required for CYCLOPS-induced NIN expression but for CYCLOPS-induced nodule organogenesis [49].

## 3. Nutrient uptake and transport across the mycorrhizal interface

### 3.1 Phosphate uptake and transport

The total phosphate (P) contents in soils can be high, but a large percentage of this P is not plant available. In addition to organic forms of P, inorganic phosphate ($P_i$) readily binds with iron, aluminum, and manganese when the pH in the soil is acidic or binds with calcium or calcium carbonate when the soil is alkaline and then becomes plant unavailable [59]. Due to the low soil concentrations, P is often a growth-limiting nutrient for plants (accounts for 0.2% of dry weight). In addition, due to the low P mobility in soils, plant P uptake leads rapidly to the development of depletion zones around the roots that further limit plant P uptake to the slow rate of diffusion. The ability of AM fungi to grow with their ERM beyond these depletion zones combined with their efficient P uptake systems is the main basis for their positive impact on P uptake and plant growth. Plants and fungi absorb P as negatively charged P ions ($H_2PO_4^-$). To take up P against the concentration gradient, high-affinity transporter proteins from the Pht1 family are required, which transport P into cells via a proton gradient generated by a plasma membrane $H^+$-ATPase [60].

During the development of arbuscules, the colonized cell undergoes a reorganization and starts to enclose newly formed hyphal branches of the arbuscule by a novel symbiosis-specific membrane, the periarbuscular membrane (PAM), which is an extension of the plant plasma membrane. The PAM plays a vital role in the nutrient exchange between the symbiotic partners and is composed of a variety of transport proteins specifically designed to facilitate the nutrient uptake from the symbiotic interface (**Figure 3**). The most extensively studied membrane proteins in the PAM are inorganic phosphate ($P_i$) transporters of the PHT1 gene family, such as MtPT4, LjPT4, and OsPT11 that were characterized in *Medicago truncatula*, *Lotus japonicus*,

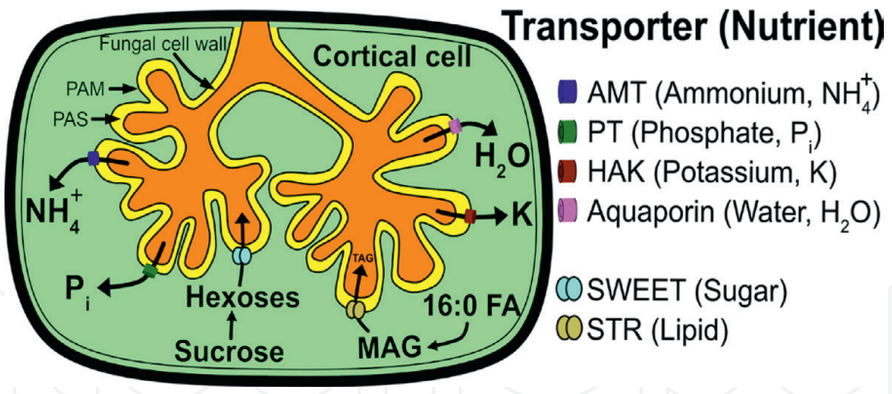


**Figure 3.**
*Nutrient exchange in the arbuscular mycorrhiza symbiosis. Nutrients such as phosphate ($P_i$), ammonium ($NH_4^+$), potassium, and other resources such as water are captured by the AM fungus and transported to the arbusculated cells in the root cortex. Transporters in the periarbuscular membrane such as phosphate transporters (PT), ammonium transporters (AMT), high-affinity $K^+$ transporters (HAK), and aquaporins (AQP) facilitate the uptake by the plant from the mycorrhizal interface. In return, the plant rewards the fungus with carbohydrates and lipids transferred into the AM interface by the transporter STR.*

or *Oryza sativa* (rice), respectively [61–63]. Most of these transporters are expressed explicitly after AM colonization and are exclusively located at the interface of the two symbionts in the PAM. Due to their unique expression pattern in response to AM fungal colonization, the expression of these AM-inducible P transporters can serve as a reliable indicator for AM colonization.

The PAM also contains P transporters that are essential for AM development but are not critical for symbiotic $P_i$ uptake. For example, OsPT11 and OsPT13 are both critical for AM development in rice, and mutations in both transporters interfere with intraradical fungal development and growth of arbuscules, but only OsPT11 plays an active role in symbiotic $P_i$ uptake. It has been suggested that OsPT13 could play a role as a $P_i$ sensor at the PAM that regulates the development of arbuscules and thereby maximizes their symbiotic transport activity [64]. Similarly, AsPT1 and AsPT4, two AM-induced $P_i$ transporters of *Astragalus sinicus*, that are also localized in the PAM, are essential for arbuscule development, but only AsPT4 is involved in symbiotic $P_i$ transport [65]. AsPT1 could encode a functional transceptor, a protein with both solute transport and receptor-like signaling activity that could mediate symbiotic $P_i$ uptake. Some AM-inducible $P_i$ transporters are also expressed in nonmycorrhizal roots. For example, the AM-induced $P_i$ transporter LjPT4 is essential for functional AM symbiosis but is also expressed in nonmycorrhizal root tips. It has been suggested that in nonmycorrhizal roots, LjPT4 integrates signals of the plant's $P_i$ status and regulates $P_i$-dependent developmental programs, such as root branching [62].

### 3.2 Nitrogen uptake and transport

Nitrogen (N) is a major driver for crop yield, and its availability significantly impacts agricultural productivity. Since N can constitute in plant tissues between 1 and 5% of the total dry matter, plants require N in large quantities, and N is the nutrient that most often limits plant growth [66]. In soils, N is present in inorganic forms, such as nitrate ($NO_3^-$) or ammonium ($NH_4^+$), and in organic form, such as urea, free amino acids, and short peptides. The availability of these N pools, however, varies considerably due to soil heterogeneity and dynamic microbial conversions of these different N forms (e.g. nitrification and denitrification) [67]. In most aerobic soils,

$NO_3^-$ is the primary type of N, whereas $NH_4^+$ can be the dominant form of N in acidic and/or anaerobic soils [68]. Plants can take up $NH_4^+$, $NO_3^-$, and organic N, but due to the strong competition with microorganisms for organic N uptake, they take up $NH_4^+$ and $NO_3^-$ in larger quantities [69]. $NO_3^-$ is very mobile in soils and readily leached, while $NH_4^+$ adsorbs onto the cation exchange complex of many soils and is only slowly released. Because plants have to also compete for $NH_4^+$ with soil microorganisms, which use $NH_4^+$ not only as an N source but also as an energy source and convert $NH_4^+$ into $NO_3^-$ via nitrification, $NO_3^-$ is a major N source for most higher plants [70].

The ERM of fungi can take up $NH_4^+$ and $NO_3^-$, but due to its higher energy efficiency, AM fungi typically prefer the uptake of $NH_4^+$ over $NO_3^-$ [71]. AM fungi have high-affinity and low-affinity uptake systems for $NH_4^+$. Fungal high-affinity uptake systems for $NH_4$ have lower $K_m$ values than plants, what allows AM fungi to take up $NH_4^+$ from soils with very low concentrations [72]. When $NO_3^-$ is available, a high-affinity $NO_3^-$ transporter is upregulated in the extraradical mycelium (ERM) of the AM fungus *Rhizophagus irregularis* [73]. However, the expression of this transporter is repressed by an external supply of $NH_4^+$ or a downstream metabolite such as glutamine [74]. Through a process that is known as N catabolite repression, $NO_3^-$ transporters in many organisms are suppressed or degraded, when more preferred N sources, such as $NH_4^+$ become available, or when the concentrations of downstream metabolites in the cells increase [75]. Due to high mobility of $NO_3^-$ in soils, the positive impact of AM fungi in N nutrition of plants is still under debate [76]. However, in *Medicago truncatula*, AM mycorrhizal growth responses can be tightly linked to mycorrhizal P but also N benefits [77]. In *MtPT4* mutants, in which the AM-induced $P_i$ transporter is not expressed, intraradical root colonization is reduced, arbuscules prematurely degenerate, and the symbiosis fails [78]. Interestingly, the premature arbuscular degeneration (PAD) is suppressed when the plant is under N starvation, indicating that AM fungi can play an important role for the N nutrition of their host [71, 79].

Labeling, enzymatic measurements, and gene expression data suggest that N that is taken up by the AM fungus is first assimilated and converted to the amino acid arginine in hyphae of the ERM, then transferred via polyphosphates (polyP) to the intraradical mycelium (IRM), and in the IRM, arginine is converted back to $NH_4^+$ via the urea cycle before it is released into the AM interface [71, 74, 80]. In several plant species, $NH_4^+$ transporters of the AMT2 family are specifically expressed during AM symbiosis and are located in the PAM [81–83]. In *Medicago truncatula*, three AMT2 family $NH_4^+$ transporters are induced during the AM symbiosis. AMT2;4 is a functional $NH_4^+$ transporter, while AMT2;3 is unable to restore growth of a yeast high-affinity $NH_4^+$ mutant on low N media, indicating that AMT2;3 plays more a sensor or signaling role [83, 84]. Interestingly, only AMT2;3 was able to suppress the premature degeneration of arbuscules in *MtPT4* or *MtPT8* knockout mutants under N deficiency.

LjAMT2;2, an $NH_4^+$ transporter in the PAM of *Lotus japonicus*, transfers $NH_3$ instead of $NH_4^+$ [83]. The expression of LjAMT2;2 is induced under severe N starvation, and LjAMT2;2 overexpression increased the biomass and N content of roots and shoots and promoted plant growth under N deficiency. Like LjAMT2;2, the AM-inducible $NH_4^+$ transporter ZmAMT3;1 is able to recruit $NH_4^+$ but transports uncharged $NH_3$ rather than ionic $NH_4^+$ [85]. It has been estimated that the ZmAMT3;1-dependent mycorrhizal N uptake pathway is responsible for 68–74% of the symbiotic N uptake of AM maize plants in pot experiments and for >30% of the postsilking N uptake of field-grown maize. Recent evidence of the putative AM-inducible nitrate transporter OsNPF4.5, which is exclusively expressed in

arbusculated cells and crucial for arbuscule development and symbiotic N uptake in rice, indicates that also $NO_3^-$ can be transferred across the AM interface [86]. In knockout mutants of *OsNPF4.5*, a 45% decrease in symbiotic N uptake and AM colonization was observed. The expression of the AM-inducible plant lysine-histidine transporter *LjLHT1.2* in arbusculated cells of *Lotus japonicus* could indicate that N, also in the form of amino acids, can be transferred across the AM interface. A significant amino acid transport across the interface, however, would be costly for the AM fungus because plant-derived carbon skeletons that were used for the biosynthesis of amino acids in the ERM would be returned to the host plant. Labeling studies, however, confirmed that there is no significant carbon flux from the fungus to the host, that amino acids are first broken down in the IRM, and that N is transferred primarily in inorganic form across the mycorrhizal interface [80]. *LjLHT1.2* is also expressed in non-arbusculated cortical cells, which suggests a more general role of LjLHT1.2 in the reuptake and recycling of amino acids in the root cortex [87].

### 3.3 Potassium uptake and transport

Potassium (K) is a necessary macronutrient that plays a key role in enzyme activation, osmotic regulation, plant cell turgor generation, cell expansion, control of membrane electric potential, and pH homeostasis [66]. Plants have evolved a variety of transporters that differ in their structure and their transport mechanisms for the uptake of K from the soil, including voltage-gated K-channels, the carrier-like families KT/HAK/KUP, HKT uniporters and symporters, and cation-proton antiporters [for review see 88]. AM fungi have also been shown to have a positive impact on the K nutrition of their host plant, but the molecular mechanisms of fungal K transport are not well understood [89, 90]. The AM symbiosis triggers transcriptional responses in *Medicago* roots under K deprivation, and these responses, including, for example, the upregulation of a $K^+/H^+$ exchanger, could activate mechanisms that help mycorrhizal plants to tolerate long-term K deprivation or facilitate the uptake of K via the MP [90]. AtCHX20, an ortholog of the $K^+/H^+$ exchanger (*Medtr7g099800.1*), plays a critical role in K homeostasis and osmoregulation [91]. In *Lotus japonicus*, the potassium transporter LjHAK is highly upregulated in AM roots [92]. A LjHAK ortholog in tomato, SlHAK10 (high-affinity potassium transporter 10), is explicitly expressed in arbusculated cells and plays a role in the mycorrhizal K uptake pathway [93]. Under low-K supply conditions, loss of function of SlHAK10 decreased mycorrhizal K uptake and AM colonization rate but did not have a negative effect on arbuscule development. By contrast, SlHAK10 overexpression increased AM colonization, plant growth, K uptake, and the soluble sugar accumulation in roots under K deficiency. An increase in K uptake could promote the carbohydrate allocation to the roots and thereby stimulate the AM colonization of the roots.

### 3.4 Water uptake and transport

In addition to elemental nutrients, water is essential to drive plant growth and nutrient uptake. It is long known that AM fungi can have a positive impact on plant-water relations and can significantly improve the tolerance of plants to drought [94]. The positive impact of AM fungi on drought tolerance has been attributed to a variety of effects, including effects on stomatal conductance, an increase in water use efficiency, reductions of the oxidative damage under drought stress, modifications in the contents of plant hormones, such as strigolactones, jasmonic acid, and abscisic

acid, improvements in plant water status by effects on hydraulic conductivity, and the activation of functional proteins, such as aquaporins [95]. For example, the AM symbiosis induced strigolactone biosynthesis under drought conditions and conferred drought tolerance in lettuce and tomatoes [96, 97]. Using a two-compartment system, it has been estimated that fungal water transport from the hyphal compartment can account for more than 30% of the water transpired by AM host plants. In addition, AM fungal hyphae were able to transport water along hyphae outside of hyphal cell membranes [98]. It has also been suggested that AM fungi increase the water uptake of plants by their positive effect on the expression of plant aquaporins [99, 100]. Aquaporins are membrane proteins that facilitate the transport of water and small solutes following an osmotic gradient. In *L. japonicus*, two putative aquaporin genes (*LjNIP1* and *LjXIP1*) are upregulated in AM plants [101]. The expression of *LjNIP1* is correlated with the expression of the AM-induced $P_i$ transporter LjPT4 in arbusculated cells. Consistent with its function as aquaporin, LjNIP1 increased the water membrane permeability, when it was expressed in yeast protoplasts.

## 4. Carbon transport in root symbioses

### 4.1 Carbon transport in the arbuscular mycorrhizal symbiosis

The fact that AM fungi are obligate biotrophs and cannot complete their life cycle without the carbon supply from their host and the observation that host plants suppress the AM colonization of their root system when nutrients are readily available has led to the overall assumption that the host plant is in control of the symbiosis [102]. However, this phytocentric view disregards the long co-evolution of both partners in the AM symbiosis (~450 million years) that allowed both partners to improve their bargaining power in the symbiosis and contributed to the evolutionary stability of mutualism in the AM symbiosis [103, 104]. In CMNs, in which multiple host plants are interconnected and share the same mycorrhizal network, AM fungi preferentially allocate nutrient resources to host plants that provide more carbon benefits [105]. The carbon supply of the host is an important trigger for nutrient uptake and transport by the AM fungus [74, 106, 107]. Similarly, host plants are simultaneously colonized by communities of AM fungi that compete with their nutrient benefits for carbon from the mycorrhizal host. Despite the fact that different AM fungi can colonize the same root on a very small spatial scale, host plants are able to distinguish between AM fungi and preferentially allocate carbon resources to AM fungi that provide higher nutrient benefits [103]. This requires that host plants can fine-tune their carbon flux to different AM fungi on a very small spatial scale and at each individual AM interface. However, our current understanding of these processes is still very limited, particularly considering that the host plant exchanges carbon with the fungus for a variety of AM benefits.

In plants, sugar transporters are classified into monosaccharide transporters (MSTs), sucrose uptake transporters (SUTs), and SWEETs (Sugars Will Eventually be Exported Transporters). Both MSTs and SUTs contain 12 transmembrane α-helices, while SWEETs are characterized by seven transmembrane domains [108]. Plant MSTs show high expression levels in AM roots, and it has been suggested that MSTs could play an important role in funneling host plant carbon to the mycorrhizal interface [108, 109]. For example, the promoter of the *Medicago* hexose transporter *MtHex1* is particularly active in inner cortical cells that are adjacent

to arbusculated cells [109]. In addition to monosaccharide transporters, there is increasing evidence that SUTs or SWEETs also play a role in the carbon allocation in mutualistic and pathogenic plant-microbe interactions. In split-root experiments with rhizobia- or AM-colonized root halves of *Medicago truncatula*, the expression levels of *MtSUT2* and *MtSUT4–1* were correlated with the amount of carbon that was allocated to the different root halves. This could indicate that both transporters play a role in the carbon transport regulation to different root symbionts [110]. High soil $P_i$ availabilities reduce the AM colonization of potato plants, but when *SoSUT1* is overexpressed the response to high $P_i$ availabilities is reduced [111]. By contrast, transgenic tomato plants, in which *SlSUT2* is downregulated, showed an increased AM colonization [112]. SlSUT2 is localized in the PAM but is presumably involved in the reuptake of sucrose from the AM interface into the host plant. A high expression of *SlSUT2* would reduce the carbon flux to the AM fungus and thereby reduce AM colonization.

While SUTs and monosaccharide transporters are symporters and require energy for the transmembrane transport of sugars, SWEETs are uniporters that can facilitate transmembrane transport in two directions and promote the diffusion of sugars along a concentration gradient. In soybeans, 52 different SWEET genes were identified [113]. Members of the SWEET transporter family show a distinct expression profile in plants and are involved in a variety of different physiological processes in plants, such as phloem transport, grain filling, floral transition, and the abiotic and biotic stress response of plants [114, 115]. In potato plants, the SWEETs *StSWEET2c, StSWEET7a,* and *StSWEET12a* are explicitly upregulated in arbusculated cells [116]. An overexpression of *SWEET7a* in potato plants increased the carbon sink strength of the roots, and these plants showed a faster colonization by AM fungi and the hemibiotrophic pathogen *Fusarium oxysporum f. sp. tuberosi* [117]. In *Medicago truncatula*, the two orthologs *MtSWEET1b* and *MtSWEET6* show high transcript levels in AM roots [110]. In addition, when the AM fungus had access to N and the carbon allocation to the AM root systems increased, *MtSWEET12*, *MtSWEET15c*, and *MtSWEET15d* showed high expression levels in AM roots but were downregulated in AM roots, when the host plant was directly supplied with N. However, many of these SWEETs have so far not been functionally characterized, and the fact that different SWEET transporters show similar expression profiles in AM roots indicates that there is some level of redundancy in the function of these transporters [110]. This redundancy likely also explains why loss of function mutants did not show an impairment in nodular or mycorrhizal function [118, 119]. For example, *MtSWEET1b* of *Medicago truncatula* is strongly upregulated in arbusculated cells and localizes to the PAM, and the overexpression of *MtSWEET1b* promoted the intraradical colonization of the host root by AM fungal hyphae, but loss of function mutants showed no impairment in the AM symbiosis [120].

Based on earlier findings, glucose seemed to be the most likely form in which the fungus takes up carbon from the AM interface [121]. The increased activities of an apoplastic acid invertase and sucrose synthase in AM roots, which could facilitate the conversion of sucrose into the glucose and fructose in the interfacial apoplast or in the cortical cytoplasm, seemed to confirm this view [122]. This is also consistent with the expression of the fungal monosaccharide transporter *MST2* in arbusculated cells of AM roots [123]. The fact that the expression of both, *MST2* and the AM-induced $P_i$ transporter *MtPt4* is correlated, suggests that the exchange of carbon for $P_i$ is tightly linked. By contrast, a reduction in the MST2 expression by gene silencing led to malformed arbuscules and reduced *MtPt4* expression.

AM fungi store carbon mainly in the form of lipids, and here, in particular, in the form of triacylglycerol and fatty acids (FAs). It has long been assumed that AM fungi use plant-derived sugars as precursors for lipid biosynthesis, and that the fungus synthesizes FAs exclusively in the intraradical mycelium [124]. However, there is growing evidence that AM fungi are FA auxotrophs, and that the plant must transfer FAs to the fungus to sustain the AM symbiosis [125, 126]. In plants, *de novo* FA biosynthesis takes place inside plastids and starts with the biosynthesis of long-chain FAs from malonyl-ACP and acetyl-CoA. Catalyzed by the acyl-ACP thioesterase-like proteins *FatA, FatB,* and *FatM, de novo* FA biosynthesis is terminated, and free FAs are exported out of the plastid. In AM roots, the expression of *FatM* is induced. Mutants in which *FatM* is impaired can still get colonized by AM fungi, but the AM development is significantly reduced [127]. In the endoplasmic reticulum, free FAs are bound to CoA to create acyl-CoA and then converted to sn-2 monoacylglycerol (ßMAGs) via RAM2. RAM2 encodes a glycerol-3-phosphate acyl transferase, and AM colonization is significantly impaired in RAM2 knockouts [128]. It has been suggested that FatM increases the release of 16:0 FAs from the plastid that are subsequently used by RAM2 to produce 16:0 ßMAG [129]. Host plants preferentially allocate carbon resources to beneficial AM fungi [103], and the finding that RAM2 expression is particularly induced in roots that are colonized with a beneficial AM fungus supports the view that RAM2 is a key element in the FA transport to the AM fungus [130]. It has been suggested that the ABCG half-transporters STR and STR2 are involved in releasing plant-derived lipids into the interfacial apoplast [129, 131]. STR and STR2 are essential for arbuscule formation, and silencing of STR/STR2 results in a stunted arbuscular phenotype [132, 133]. Both are localized in the PAM and function as heterodimers. Another transporter that is likely involved in lipid transport in *Medicago truncatula*, is ABCG3/WBC5. It has been suggested that ABCG3/WBC5 could be part of the RAM1-regulated lipid export pathway [125]. Together with *RAM2*, *MtABCG3* is strongly induced in mycorrhizal roots and regulated by the GRAS-domain transcription factor RAM1 (required for Arbuscular Mycorrhization 1) [125, 134].

### 4.2 Carbon transport in the symbiosis of legumes and rhizobia

Biological nitrogen fixation (BNF) by rhizobia is an energetically costly process. At least 16 molecules of ATP and 8 low potential electrons are necessary to convert $N_2$ into $NH_3$ [135]. Plants must provide rhizobia with a constant flow of energy in the form of reduced carbon compounds to maintain BNF activity. Root nodules constitute only a small fraction of the total biomass of a legume plant, but they can consume more than 25% of the total photosynthates of the plant. Sucrose is the main transport sugar that is translocated through the phloem to root nodules and to the other sink organs of the plant. However, how carbon is funneled to the root nodules and how this process is regulated is still largely unknown. For example, the nodule-specific sucrose transporter *MtSWEET11* of *Medicago truncatula* is expressed in infected root hair cells and in the meristem, invasion zone, and vascular system of nodules. However, although *MtSWEET11* seems to play a role in the sucrose distribution in root nodules, it is not essential for BNF [118]. The sucrose uptake transporter *GmSUT1* shows a high expression in the nodules of soybeans and is particularly localized in the peripheral fixation zone and the vascular bundles [136]. Since *GmSUT1* overexpression increased nodule number and plant N content, it has been suggested that GmSUT1 could play a role in funneling sucrose to the root nodules.

The symbiosome membrane (SYM) is the site of nutrient exchange between plants and rhizobia and is energized through the activities of $H^+$-ATPases that pump protons into the symbiosome space and provide both the plant and bacteroids with the necessary proton motive force to take up nutrients from the symbiosome space [137]. Among 197 proteins that were identified in the SYM of soybeans, were proteins involved in metabolism, protein folding and degradation, membrane trafficking, and solute transport, such as putative transporters for sulfate, calcium, hydrogen ions, peptide/dicarboxylate, and nitrate [138]. Mutations that interfere with dicarboxylate transport across the SYM interrupt $N_2$ fixation, and it has been suggested that $C_4$-dicarboxylates are the primary carbon source for bacteroids in active root nodules. The SYM has a well-characterized dicarboxylate transport system (Dct) encoded by the three gene loci *dctA*, *dctB*, and *dctD* [139]. While *dctB* and *dctD* encode a two-component regulatory system that responds to the presence of $C_4$-dicarboxylates, *dctA* encodes the structural protein for $C_4$-dicarboxylate transport. Succinate, malate, fumarate, aspartate, and oxaloacetate have been discussed as the most important substrates for rhizobial Dct systems.

Bacteroids transfer N primarily in the form of $NH_4^+$ to the plant but also secrete significant amounts of the amino acids aspartate and alanine [140]. $NH_3$, the product of BNF, is either transferred via passive diffusion across the bacteroid membrane or through the nodulin 26 channel [141]. Nodulin 26 is a member of the aquaporin superfamily but also shows an ammonia permease activity that is favored over its aquaporin activity [141]. Due to the low pH in the symbiosome space, $NH_3$ is protonated here to $NH_4^+$ and then taken up by the host via a cation channel that is permeable to $NH_4^+$ [142].

### 4.3 Regulation of nodulation and mycorrhizal colonization

Due to the high carbon costs of both root symbioses, plants are under high selective pressure to tightly control nodulation and AM colonization. N and P deficiency in plants stimulates the colonization of plants by rhizobial or AM fungal partners, while a high availability of N or P, or the inability of partners to provide benefits to the host plant, promotes the premature senescence of symbiotic organs [143, 144]. The interaction between the partners in the RL symbiosis is based on carbon-nitrogen trade-offs. Since the plant cannot determine the BNF efficiency of rhizobia during the recognition and root entry stages, the plant needs to sanction low-benefit rhizobia later [145, 146]. Legumes have control over the carbon supply and can influence the success of their symbiotic partners. In natural soils, non-fixing rhizobia strains are rare, but intermediate fixers are common. When the plant can choose between an intermediate fixer and a more effective strain, the plant will "sanction" the intermediate fixer by limiting its carbon supply, what results in smaller nodules with fewer viable bacteria. By contrast, when the only alternative is a non-fixing rhizobia strain, plants will not sanction intermediate fixers [147]. Environmental variation can help to explain why low- and high-benefit rhizobia still coexist. In an N-rich environment, the host might be less strict, thus allowing low-benefit strains to proliferate [148]. Low-benefit rhizobia might also be able to escape host plant sanctions by forming mixed nodules with high mutualistic rhizobia strains [149].

Arbuscules have only a short lifespan and are only functional for 2–3 days, before they become senescent and collapse [150]. This short life cycle seems like a waste of limited resources but allows plants to regulate their AM colonization. Plants can also induce a premature arbuscular degeneration (PAD) [84]. PAD has been discussed as

one mechanism by which host plants can sanction low-benefit AM fungi or are able to reduce the AM colonization under high P conditions [79, 151]. Host plants can distinguish between high- and low-benefit AM fungi and allocate carbon resources accordingly [103, 130]. However, low-benefit AM fungi are able to persist in natural environments, and host plants tolerate the infection by low-benefit AM strains, to reduce their dependency on a single AM fungus for nutrient uptake.

However, plants not only control the colonization with low-benefit rhizobia but also limit nodule formation with high-benefit partners by the long-distance (systemic) autoregulation of nodulation (AON) pathway [152]. In response to rhizobial infection, a subset of genes encoding CLAVATA3/Embryo Surrounding Region (CLE) peptides are activated [153]. CLE peptides consist of 12–13 amino acids, secreted as signaling peptides from the C-terminal region of preproproteins. It has been reported that AON-related CLE peptides from the root negatively affect the number of nodules by inhibiting a leucine-rich repeat receptor-like-kinase (LRR-RLK) in the shoot, known as SUPER NUMERIC NODULES (*SUNN*) in *Medicago truncatula*, HYPERNODULATION ABERRANT ROOT FORMATION (*HAR1*) in *Lotus japonicus*, NODULE AUTOREGULATION RECEPTOR KINASE (*NARK*) in soybean, and SYMBIOSIS 29 (*SYM29*) in peas [154–157]. The perception of the root-derived signals by LRR-RLK in the shoot generates a shoot to root signal that inhibits further colonization. It has been suggested that this shoot-derived inhibitor could be a cytokinin [158, 159]. Currently, our understanding of the downstream targets of the AON pathway in the roots is limited. Compared to the wild type, supernodulating SUNN mutants, for example, showed an increase in polar auxin transport from the shoot to the root, and it has been suggested that higher auxin levels could be responsible for the interruption of AON [160]. In transgenic roots of *Medicago truncatula* that ectopically express either *MtCLE12* or *MtCLE13*, the early nodulation marker *MtENOD11* was not activated, suggesting that the systemic AON pathway could inhibit Nod factor signaling [153].

A prior AM colonization of one root half in a split-root system significantly reduces the AM colonization of the second root half, and there is evidence that the systemic autoregulation of mycorrhization (AOM) has similarities to the AON pathway [161, 162]. For example, hypernodulating mutants such as sunn, nark, har1, and sym29, also show a hypermycorrhizal colonization phenotype. However, whether other AON genes or mobile signals (CLE) are also involved in AOM is not yet known. However, the fact that prior nodulation in split-root studies can systemically suppress AM colonization and vice versa suggests that the same shoot-derived inhibitor is involved in AON and AOM.

## 5. Tripartite interactions of legumes

Our current understanding of the RL or AM symbiosis is mainly based on single plant/single symbiont studies, but field grown legumes form tripartite interactions, and are simultaneously colonized by both AM fungi and rhizobia [110, 163]. The inoculation with both root symbionts can lead to synergistic benefits, and plants can gain more from tripartite interactions than from single inoculations with either symbiont [163–165]. The rhizobial nitrogenase complex requires 16 ATP to fix one $N_2$ molecule, and consequently rhizobia require an adequate P supply for efficient BNF. Nodules act as strong P sinks in legume root systems to provide sufficient P resources for optimum BNF [110, 166]. Nonmycorrhizal soybean plants have lower

nodule numbers and weights and show particularly under low P supply lower N fixation rates [163, 167]. The positive effect of the AM symbiosis on the P uptake of the plant has therefore been discussed as the primary reason for the stimulation of BNF in AM legumes [163]. In addition, AM fungi can also supply the plant with other microelements that are essential for $N_2$ fixation, including zinc, iron, manganese, and molybdenum [168, 169].

However, antagonistic responses have also been described, and the prior inoculation with either rhizobia or AM fungi can also suppress the root colonization by the other partner [170]. Whether the plant shows antagonistic or synergistic growth responses after dual inoculation depends on the environmental context [164], and the compatibility between symbiotic partners [165, 171]. As long as the root symbionts provide complementary rewards to the host plant, and the benefits outweigh the costs of these interactions, synergistic growth responses are more likely [172]. Rhizobia and AM fungi, however, are also competitors for the same resource from the host plant, and since both interactions are costly, the plant must be able to allocate carbon resources to both root symbionts according to their benefits. For example, an AM fungal partner is a stronger competitor for host plant carbon when it is able to provide N to the host, or when the plant is grown under high N but low P availabilities [110]. Our current understanding of the molecular mechanisms that control the carbon allocation from the host plant to individual root symbionts is very limited. Considering, the role that host plant carbon plays as an important trigger for symbiotic functioning, a better understanding of these processes is critical, because it may be key to improve the resource exchange between plants and symbionts and ultimately enhance productivity of agronomically important legumes [74, 105, 173, 174].

## Conflict of interest

Authors declare no conflicts of interest in this work.

## Author details

Debatosh Das, Salina Tripathi, Prema Mutyala, Dhruv Aditya Srivastava and Heike Bücking*
Division of Plant Science and Technology, University of Missouri, College of Agriculture, Food and Natural Resources, Columbia, MO, USA

*Address all correspondence to: heike.buecking@missouri.edu

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