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Mechanisms Underlying Establishment of Arbuscular Mycorrhizal Symbioses

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Abstract

Most land plants engage in mutually beneficial interactions with arbuscular mycorrhizal (AM) fungi, the fungus providing phosphate and nitrogen in exchange for fixed carbon. During presymbiosis, both organisms communicate via oligosaccharides and butenolides. The requirement for a rice chitin receptor in symbiosis-induced lateral root development suggests that cell division programs operate in inner root tissues during both AM and nodule symbioses. Furthermore, the identification of transcription factors underpinning arbuscule development and degeneration reemphasized the plant's regulatory dominance in AM symbiosis. Finally, the finding that AM fungi, as lipid auxotrophs, depend on plant fatty acids (FAs) to complete their asexual life cycle revealed the basis for fungal biotrophy. Intriguingly, lipid metabolism is also central for asexual reproduction and interaction of the fungal sister clade, the Mucoromycotina, with endobacteria, indicative of an evolutionarily ancient role for lipids in fungal mutualism.

INTRODUCTION

Arbuscular mycorrhizal (AM) symbiosis is an extremely prevalent interaction between members of the Glomeromycotina fungi and roots of most terrestrial plants, including important crops. AM symbioses are considered mutualistic because of the significant nutritional benefit both partners derive from each other; up to 20% of carbon assimilated by plants can be fed to the fungus (68) in exchange for fungus-supplied minerals. Inorganic phosphate (Pi) delivery is particularly pronounced, with some fungi delivering 70–100% of the plant's overall Pi acquisition (152, 153, 184). AM symbioses can thus have a huge impact on plant performance and broader ecosystem functioning (172), impacts that have been recognized for their potential value in sustainable agroecosystems (32, 145).

AM symbiosis first evolved approximately 400–450 million years ago (9, 134, 135), coinciding with the emergence of the early diverging land plants (77), which has led to the widely shared view that association with soil-exploring fungi may have been instrumental for the transition of plants from water to land (**Figure 1**) (114, 122, 127). Indeed, extant basal liverwort species engage in AM symbiosis, although simultaneous interaction with other ancient beneficial fungi belonging to the more basal Mucoromycotina subphylum is often observed (9, 35, 175). In fact, the earliest diverging clade within the liverworts associates exclusively with Mucoromycotina (9). This suggests a transition in fungal dominance from Mucoromycotina to AM fungi (AMF) during vascular plant diversification, although the factors driving this shift remain unclear. Conceivably, the two types of fungi could differ in their capacity to support the mineral demands of their hosts, which may have selected for the more efficiently nourishing fungal partner as plant body complexity and size increased during evolution. Whatever the driving force behind the preference, the perpetuation of AM symbiosis explains today's ubiquitous distribution across the plant kingdom. Later in the evolutionary history, losses in the ability to form AM relationships resulted in a minority of nonmycorrhizal plant species. This loss appears to be genetically determined, as illustrated by recent phylogenomic studies on host and nonhost genomes, which identified a suite of genes present in mycorrhizal plant species and absent from nonmycorrhizal plant species (14, 28, 34). Indeed, reverse genetics analyses of AM-associated genes suggest that the majority of the AM-associated genes play crucial roles during the establishment of symbioses, providing a pool of genes that can be drawn from to elucidate the molecular mechanisms underlying AM symbioses (14).

The development of an AM symbiosis is a highly dynamic process that can be divided into distinct stages: (*a*) presymbiotic communication, (*b*) contact and penetration, (*c*) outer cortex invasion, (*d*) arbuscule formation, and (*e*) vesicle and spore formation (**Figure 2**). Although these stages occur consecutively at individual infection sites on a whole-root level, AM symbiosis is a nonsynchronous process marked by the concomitant presence of all the above-described steps. Orchestrating the complex development of this alliance therefore requires elaborate and well-coordinated signaling mechanisms. Elucidation of the molecular underpinnings of AM symbioses has intrigued molecular geneticists for more than a decade, with the discovery of the first functionally required gene (33, 157) fueling research into this area. This review discusses the emerging signaling concepts from precontact recognition in the rhizosphere to the stage of extreme plant-fungal intimacy, the arbusculated cell (for complementary reviews, see 98, 99).

PRESYMBIOTIC COMMUNICATION

Prior to physical contact, plants and AMF release a bouquet of diffusible molecules into the soil to attract their symbiotic partner and initiate cellular reprogramming for infection. Perception of the signaling molecules and cellular transmission of this information require plant and fungal receptor proteins to activate signaling cascades that enable launching the appropriate symbiosis responses.

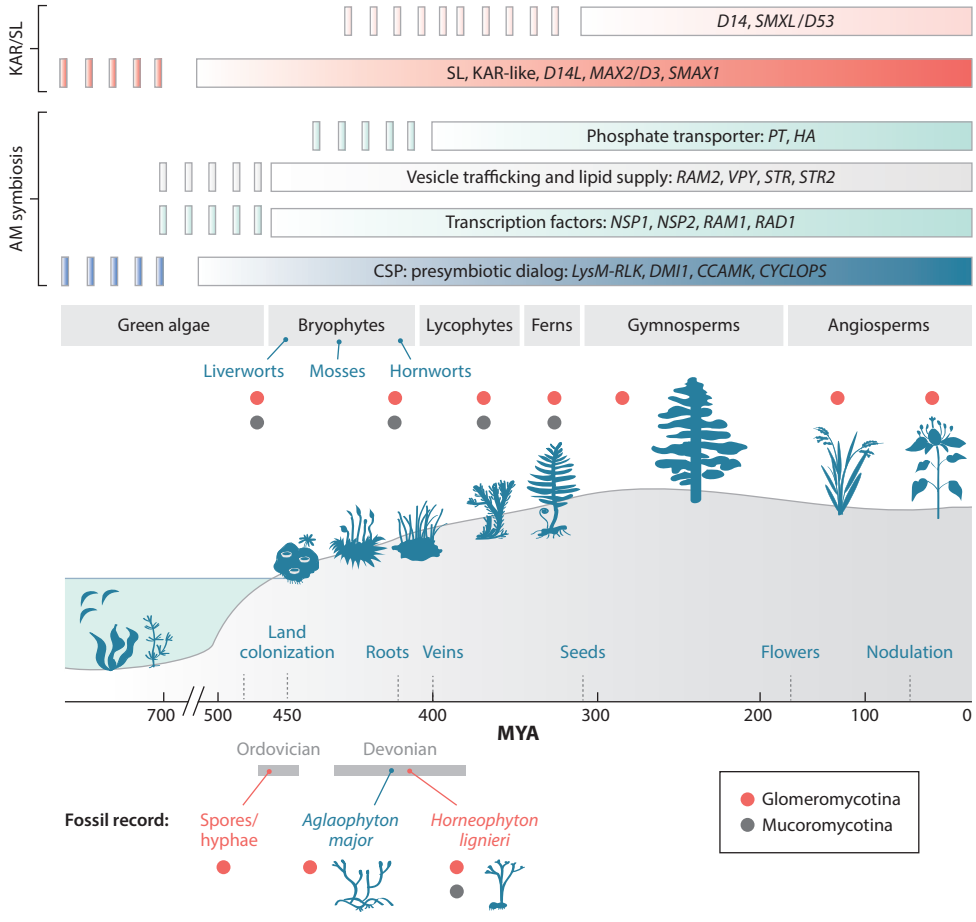

Figure 1

Arbuscular mycorrhiza and evolution. Arbuscular mycorrhizal (AM) symbiosis represents an ancient and ubiquitous beneficial mutualism between plants and fungi belonging to the Glomeromycotina (*red circle*). Fossils featuring the extinct plant species *Aglaophyton major* or *Horneophyton lignieri* date back to the early Devonian era, 400–450 million years ago (MYA), contained fungal structures resembling arbuscules (9, 134, 135). AM symbioses are widespread from the basal to the higher land plants, except for mosses. Fossil records and extant earliest diverging land plants display intracellular colonization by members of the Mucuromycotina (*gray circle*), another ancient fungal lineage (9, 35, 158). Consistent with the ancient evolutionary origin of the AM symbiosis, phylogenomics studies revealed a core set of genes associated with the AM trait, including transcription factors, transporters, and genes encoding proteins for intracellular fungal accommodation (14, 28, 34). Nodulation developed by co-opting genetic programs of the ancient AM symbiosis (122). Genes associated with butenolide signaling are present across the plant kingdom, even in green algae (27, 178). Thus, AM symbiosis–associated genes have an ancient evolutionary origin, with retained roles in modern plant species. Abbreviations: CSP, common symbiosis signaling pathway; KAR, karrikin; SL, strigolactone.

Root Exudates Stimulate Arbuscular Mycorrhizal Fungi for Symbiosis

Plant roots release a plethora of metabolites into the soil, many of which serve chemical communication with soil microbes (171). Host root exudates activate AM fungal metabolism and, likely as a result, distinct hyphal branching patterns that increase the chance for encountering the host (8, 17). More than 10 years ago, strigolactones (SLs) were identified as key components

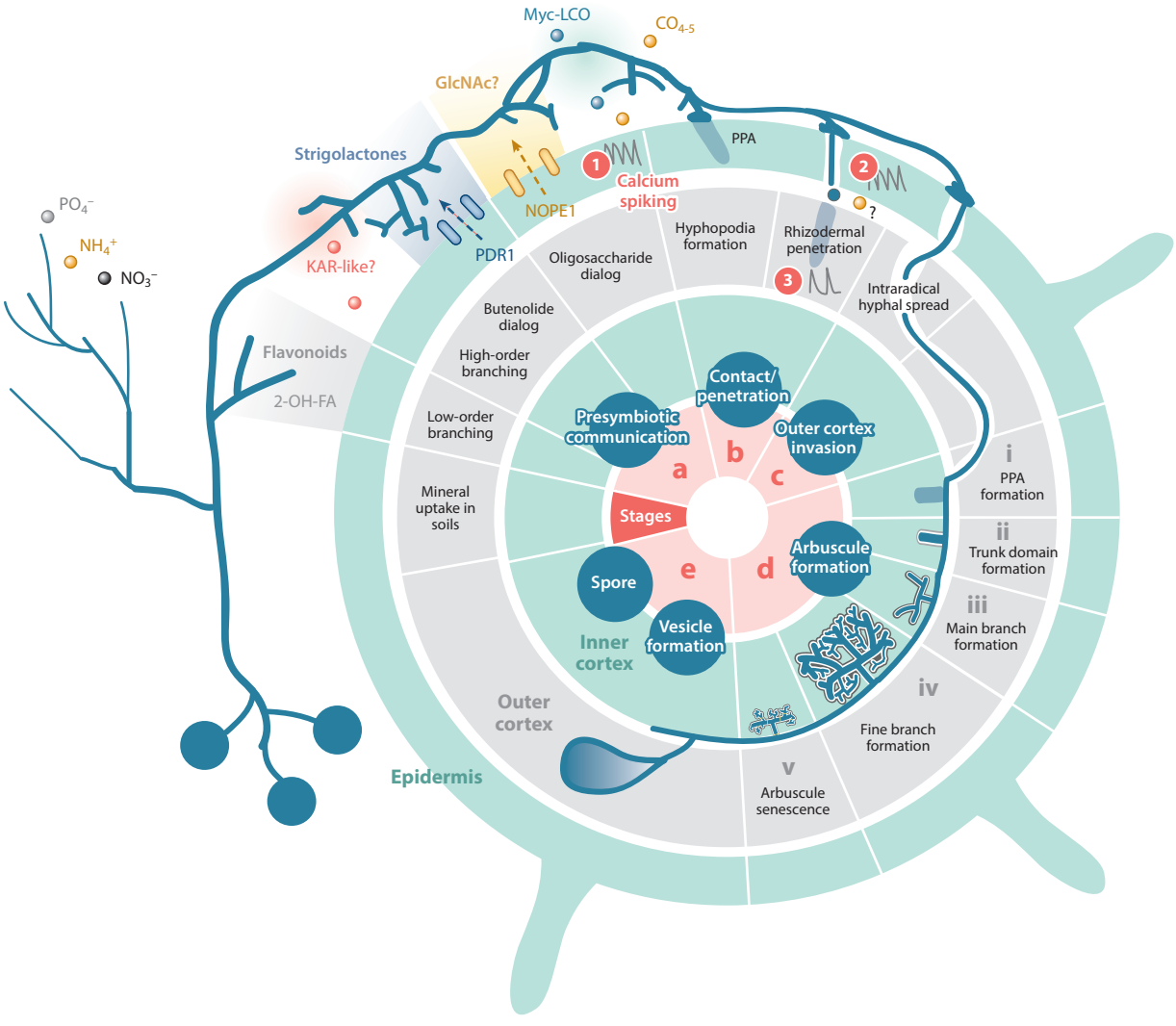

Figure 2

Life cycle of AM symbiosis. Under optimal abiotic conditions, fungal spores germinate and navigate toward the host by root exudates. (*a*) Prior to contact, both organisms detect diffusible signals released by the prospective partner, namely butenolides (reviewed in 178) and oligosaccharides (reviewed in 90, 194). Perception of fungal oligosaccharides such as chitotetraose/chitopentaose (CO_{4-5}) and mycorrhiza-lipooligosaccharides (Myc-LCOs) triggers nuclear calcium spiking in the rhizodermis to activate the common symbiosis signaling pathway (CSP) (steps ①, ②, and ③), which regulates gene expression required for rhizodermal penetration. (*b*) Fungal attachment structures, called hyphopodia, form on the cell surface while the host cell produces a prepenetration apparatus (PPA) to intracellularly accommodate the fungus (45). (*c*) Fungal hyphae grow toward the inner cortex to initiate arbuscule formation (46). (*d*) Arbuscule development can be divided into five substages featuring development and collapse of arbuscules (stages i–v). (*e*) Successful arbuscule formation enables carbon uptake in exchange for mineral nutrients delivered by the fungus, leading to the formation of nutrient storage vesicles and daughter spores. Abbreviation: KAR, karrikin.

of root exudates (reviewed in 178), acting as potent stimulants of fungal metabolism and hyphal fine branching (1). Export of SLs into the rhizosphere involves the ABC-transporter Pleiotropic drug resistance1 (PDR1), producing a concentration gradient that provides a positional cue for guiding the beneficial fungus toward the host root (84). Notably, this strategy has been co-opted by parasitic plants where SLs are essential for the induction of seed germination and subsequent guidance of the radicle to the host (11). In addition, SLs are important endogenous plant growth regulators that modulate both the shoot and root architecture of higher plants (49, 103, 170) as well as protonema morphology in moss (128). Therefore, SLs may have played a central role not only in plant development but also in contributing to symbiotic rhizosphere signaling from bryophytes to angiosperms.

SLs are produced from carotenoids via a biosynthetic pathway that includes the intermediate precursor carlactone. Under AM symbiosis–promoting, low-Pi conditions, mRNA levels of both the SL biosynthetic β-carotene isomerase-encoding *Dwarf27* and the SL exporter *PDR1* gene increase, linking the plant's nutritional status with SL-mediated attraction of beneficial fungi (84, 94). However, the pronounced release of SLs from roots is not necessarily uniform, with SLs found to be below detection levels in root exudates of some mycorrhizal plant species such as sunflower and black oat (76, 169). Instead, these species accumulate carlactones, some of which have been recently reported to also promote AM hyphal branching (109) and may therefore contribute to presymbiotic signaling alongside SLs. The fungal perception machinery for plant SLs or carlactones has not yet been identified but is of major importance for the understanding of SL-related mechanisms that prepare the fungus for symbiosis.

Intriguingly, a global transcriptional analysis of *Rhizophagus irregularis* in response to treatment with rice root exudates recently revealed the rapid activation of fungal genes encoding signaling components, most notably kinases (111). This signature was absent when *R. irregularis* was treated with root exudates of a rice mutant lacking the N-acetylglucosamine (GlcNAc) transporter No perception 1 (NOPE1), which instead induced genes associated with stress responses. The differential transcriptional response of *R. irregularis* therefore suggested that NOPE1 influences the release of signals related to GlcNAc that are required for conditioning the fungus for root colonization. *R. irregularis* presymbiotically expresses a homologous GlcNAc transporter gene at low levels (111) that might be able to transport similar substrates as NOPE1. Importantly, fungus treated with *nope1* root exudates still exhibited wild-type levels of hyphal branching, reflecting that despite the fungal inability to invade the root, the response to SLs was not impaired (111). Taken together, host plants exude a cocktail of signaling components that stimulate distinct responses in AMF and prime the fungus for subsequent interaction with the host.

Arbuscular Mycorrhizal Fungal Precontact Signals

Microbes release a diverse range of compounds into their environment to facilitate intra- and interspecies communication. AMF are no exception, with *R. irregularis* exuding the small protein SL-induced putative secreted protein 1 (SIS1) into the rhizosphere upon SL treatment (168). Although the exact mechanism of SIS1 functioning is currently unknown, downregulation of *SIS1* transcripts by host-induced gene silencing (HIGS) was correlated with overall reduced root infection and with the development of smaller arbuscules, indicating a role for SIS1 in promoting fungal root colonization (168). SIS1 thus represents the first example of a proteinaceous AM fungal rhizosphere signal, which is functionally required for root colonization.

Chemical communication in microbes frequently involves oligosaccharide signals. AMF secrete at least two types of oligosaccharides, mycorrhizal-lipochitooligosaccharides (Myc-LCOs) and short-chain chitooligosaccharides (COs), namely chitotetraose (CO_4) and chitopentaose

(CO_5) (**Figure 2**) (43, 101). These signals share the same β-1–4-linked GlcNAc backbone; however, although COs are built of simple repeats, LCOs have a more complex structure, as they are further modified with oleic or palmitic acid at the N terminus and can also occur in the O-sulfated or nonsulfated form at the C terminus (101). Both Myc-LCOs and COs are chemically closely related to other well-known microbial signals: (*a*) the Nod factors of symbiotic rhizobium bacteria (Nod-LCOs), which are indispensable for the establishment of the interaction between rhizobia and legumes (reviewed in 117), and (*b*) the potent microbe-associated molecular patterns (MAMPs) chitoheptaose (CO_7) and chitooctaose (CO_8), which upon recognition by the plant lead to pattern-triggered immunity (PTI) (19, 71, 93, 106).

The structural similarity of these microbial GlcNAc-based molecules has therefore prompted the question of whether they are sufficiently different to achieve signaling specificity for the interaction with AMF relative to rhizobia or pathogenic fungi. In legumes, common responses to Myc-LCOs and Nod-LCOs are observed, which include root hair branching, perinuclear calcium spiking, AM promotion, and lateral root induction (43, 82, 101, 116, 159, 190). One of the most dramatic differences is the Nod-LCO-induced molecular and cellular responses that lead to organogenesis of the nodule (117). Conversely, the development of AM symbiosis does not cause profound changes in root morphology. However, an increase in lateral root formation in response to AM fungal inoculation has frequently been reported (55, 101, 110, 116, 159). As germinating spore exudates (GSEs) of AMF contain a mix of the GlcNAc signals, their relative proportions might be important to specify AMF (COs and Myc-LCOs) as opposed to rhizobia (Nod-LCOs) and detrimental fungi (COs). Interestingly, exposing germinating spores of *R. irregularis* to SLs specifically enhanced fungal secretion of CO_4 (43), supporting the view that the ratio of fungal GlcNAc derivatives might be of diagnostic relevance.

Plant Recognition of Arbuscular Mycorrhizal Fungi

Perception of GlcNAc oligosaccharides is mediated at the plant cell surface by receptor-like kinases (RLKs) containing lysin motifs (LysMs) in their extracellular domain that are capable of GlcNAc binding (reviewed in 90, 194). Legumes engage in root endosymbiosis with both AMF and rhizobia, and research in *Lotus japonicus* and *Medicago truncatula* revealed that a heteromeric LysM-RLK complex comprising kinase-active *L. japonicus* Nod factor receptor 1 (LjNFR1)/*M. truncatula* Lysin motif receptor-like kinase 3 (MtLYK3) (hereafter, NFR1) and kinase-inactive LjNFR5/*M. truncatula* Nod factor perception (MtNFP) (hereafter, NFR5) binds Nod-LCOs at physiological concentrations. This binding then leads to rapid nuclear calcium spiking that activates the common symbiosis signaling pathway (CSP), nodule organogenesis, and the initiation of the bacterial root infection process (3, 16, 89; for recent reviews, see 90, 100, 131, 194). However, revisiting the legume Nod-factor receptor mutants to determine their roles in AMF perception revealed at most only a subtle, if any, altered phenotype of *Ljnfr1* or *Mtlyk3* and wild-type colonization for *Ljnfr5* mutants (132, 190). The absence of a strong AM phenotype can be explained by the genetic redundancy of the LysM-RLKs in the legumes, with 21 and 26 LysM-RLKs in *L. japonicus* and *M. truncatula*, respectively, as opposed to 5 and 10 LysM-RLKs in the genomes of *Arabidopsis thaliana* and rice, respectively (5, 95, 192). The expansion of the LysM-RLKs in the legumes might reflect the legumes' increased ability for a more fine-tuned recognition of potential microbial interactors. Remarkably, disruption and RNAi-mediated downregulation of the rice homolog of *NFR1*, *Chitin Elicitor Receptor Kinase 1* (*CERK1*), resulted in significantly reduced fungal colonization and abnormal infection structures (24, 108, 190). In contrast, mutation of the rice *NFR5* homolog had no effect on fungal colonization (107), although RNAi or virus-induced gene silencing (VIGS)-mediated downregulation of *Parasponia*

andersonii and *Solanum lycopersicum NFR5* homologs produced strong AM colonization phenotypes (18, 118). This suggests that recognition of AMF might involve different LysM-RLKs in different plant species. However, the formal possibility that more than the one *NFR5* target gene had been silenced in *P. andersonii* and *S. lycopersicum* cannot be excluded.

In rice, CERK1 is known for its involvement in PTI by creating a receptor complex with either Chitin elicitor binding protein (CEBiP) or Lysin-motif-containing protein 4 (LYP4)/LYP6 to perceive long-chain COs, peptidoglycan, and lipooligosaccharides (30, 90). The level of wild-type AM colonization in *cebip* mutants suggested that the *cerk1* phenotype was not due to perturbation of known plant immunity signaling pathways but rather to its direct role in AMF compatibility (108). Rice CERK1 lacks binding affinity for CO_4 and CO_5 (12, 92) despite containing three LysM domains in the extracellular domain. It is therefore likely that additional proteins, other than CEBiP and NFR5, interact with CERK1 for AMF ligand perception (108). The recent discovery of *L. japonicus* CERK6 as a chitin receptor required for defense signaling but not for endosymbiosis and able to bind long-chain but not short-chain COs (polymerization degree >6) revealed that Nod-LCO recognition by the NFR1/NFR5 receptor complex is uncoupled from chitin perception by CERK6 in legumes (12). This finding reiterated that although individual legume LysM-RLKs mediate the identification of distinct GlcNAc oligosaccharides from complex exudate mixtures, the receptors for Myc-LCOs and $CO_{4\text{-}5}$ remain elusive.

Similar to root nodulation symbioses, an early and rapid root response to AMF signals is the induction of nuclear calcium spiking (82). Using calcium oscillation traces as a readout for perception, a large body of literature has documented the sensitivity of different cell types, root types, plant species, and genotypes to treatment with single or combined AM fungal GlcNAc signals; the resulting picture is complex (for recent reviews see 90, 194). Interestingly, the application of GSEs, containing the complete complement of fungal GlcNAc signals, failed to induce calcium oscillations in rice *cerk1*. Wild-type-like oscillations were, however, observed in *cebip* and *nfr5* mutants (21). The ability to trigger the calcium spiking signature therefore correlated with the wild-type root colonization phenotypes of *cebip* and *nfr5* (107, 108). The complete absence of calcium spiking in *cerk1* mutants (21) did not, however, correlate with the reduced level of fungal colonization of the mutant (24, 108, 190). Remarkably, *cerk1* mutants exhibited a loss of lateral root induction in response to inoculation with *R. irregularis* (24), defining a new and central role for rice CERK1 in symbiosis-triggered root development. Thus, CERK1 signaling links calcium spiking with induction of lateral root development and furthermore with the activation of cell division, which is reminiscent of the role of Nod-factor receptors in nodule meristem initiation. Nevertheless, the relaxed requirement for calcium spiking in AM symbiosis (21), at least in rice, suggests that additional mechanisms determine the progression of symbiosis development.

The observation that the karrikin receptor Dwarf 14–Like (D14L) is indispensable for AMF perception has been a game-changing discovery (56). Lack of *D14L* rendered rice mutants incapable of perceiving AMF as reflected by (*a*) the absence of a rapid transcriptional response to GSE and (*b*) the missing plant-fungal contact formation (56). D14L is the homolog of *A. thaliana* Karrikin Insensitive2 (KAI2), which belongs to the α/β-fold hydrolases and is the evolutionarily older paralog of the SL receptor D14. Karrikin (KAR) is a smoke-derived compound responsible for the restoration of vegetation post-wildfire (36). Both D14 and D14L bind the butenolide ring of SL and KAR, respectively (reviewed in 178). KAI2 is known to have diverse roles in photomorphogenesis, seed germination and seedling growth, and drought resistance (88, 160, 179, 180). Both D14 and D14L genetically interact with the F-Box E3 ligase More Axillary Growth 2 (MAX2) (113, 156). The rice homolog of *MAX2*, *Dwarf3* (*D3*), is required for shoot branching inhibition via the SL pathway (66, 193) as well as for the early communication with AMF (56, 186). In *Arabidopsis* KAR signaling, a *max2* suppressor screen identified Suppressor of MAX2-1 (SMAX1)

as a negative regulator and possible target for MAX2-mediated degradation (155). However, the involvement of SMAX1 in AM colonization remains unknown.

Although D14L can directly bind karrikin, the ligand required for D14L during AM symbiosis is still unknown. The developmental phenotypes of *Arabidopsis kai2* and the activation of a karrikin-reporter by *Arabidopsis* leaf extracts have led to the suggestion that D14L recognizes an endogenous KAR-like butenolide (KL) that regulates plant development (25, 37, 161). Likewise, an unknown endogenous compound might regulate the plant's capacity to recognize AMF by activating a receptor at the cell membrane. However, there is also the intriguing alternative that a KAR-like butenolide is produced by AMF. The endophytic fungus *Aspergillus terreus*, for instance, synthesizes a range of butenolides during both saprotrophic and in planta growth, some of which have inhibitory effects on the production of host reactive oxygen species (ROS) (53, 115, 123). Therefore, fungi have the capacity to produce compounds related to the known D14L ligand while engaging with a plant partner.

Interestingly, rice mutant plants lacking functional *D14L* despite the absence of AMF interaction still displayed lateral root induction at equivalent levels to wild-type plants (24). Lateral root promotion thus occurred independent of D14L, whereas root colonization required D14L, thereby D14L uncoupled AM symbiosis establishment from fungus-induced remodeling of the root system. Furthermore, as mutants of *D14L* do not support any physical interaction with the fungus the stimulation of lateral root production must be triggered presymbiotically. The immediate rice signaling response to GSE treatment observed in wild type (within 24 h post-treatment) was absent in *D14L* mutant plants (56), pointing toward a slower change in the activation genes associated with lateral root induction downstream of CERK1 signaling.

Together, insights obtained over recent years into the molecules and proteins that mediate presymbiotic plant-fungal communication in AM symbiosis have led to the emergence of common chemical concepts for the dialog between the two organisms. These involve GlcNAc derivatives, where in addition to the intensely studied AM fungal oligosaccharide signals, plants may use related compounds to presymbiotically prime the fungus for the alliance (111). Analogously, in the facultative human pathogen *Candida albicans*, host-exuded GlcNAc triggered the switch from the coccal yeast to the invasive hyphal form (reviewed in 81). An additional common chemical concept is butenolide signaling that includes SLs and KL molecules. This could be proposed as a broader theme, building on the hypothesis that D14L symbiosis signaling is initiated by binding to a butenolide ligand of AM fungal origin. It is now important to identify the bioactive GlcNAc- and butenolide-related molecules that bind NOPE1 and D14L, respectively, to understand the languages spoken.

CONTACT AND PENETRATION

Hyphopodia Formation

Following reciprocal recognition in the rhizosphere, AM fungal hyphae form foot-like attachment structures, called hyphopodia, on the surface of the root epidermis. SL stimulation of fungal hyphae is not a prerequisite, as nonelicited AMF develop reduced but normal hyphopodia on SL biosynthetic mutants prior to colonization of the root (49, 57, 80). The differentiation of fungal hyphopodia has long been suggested to result from contact recognition, as hyphopodia developed on isolated cell wall fragments from carrot host but not from sugar beet nonhost roots (112). These observations on dead plant material suggested that precontact signaling was not essential. However, the strong defects in hyphopodia formation in the maize and rice *nope1* mutants (111)

and the complete absence of hyphopodia from rice lines lacking functional *D14L* (56) collectively revealed that successful presymbiotic communication is a prerequisite for hyphopodia formation. Surface properties of the rhizodermal cell layer likely play an additional role. Interestingly, the spatially restricted induction of a rice lipid transfer protein (Ltp) in regions of the root where AM fungal hyphopodia and initial cell penetration had occurred (10) led to the proposal that fatty acids (FAs) are delivered to initial fungal contact sites. Although no functional studies for Ltp are available, the possible transfer of FAs from the plant to the fungus is particularly intriguing in light of the recent finding that AMF are FA heterotrophs and are nourished with FAs by the plant host (see below; 13, 70, 75, 97, 182). Although it is clear that the host plant secretes signals that condition AMF to prepare for contact formation, the contribution of rhizodermal cell wall properties to contact recognition remains to be investigated.

Rhizodermal Invasion

Host plants actively engage in accommodation of AMF by creating a tube-like intracellular infection structure called the prepenetration apparatus (PPA), which requires cellular reorganization and symbiosis-specific gene expression (6, 44, 45, 129). Host cell preparation for fungal invasion is a profoundly different response than the papilla formation seen in plant cells in response to appressoria formation of pathogenic fungi, which interferes with cellular entry of the fungus (146). The PPA is formed as an invaginated plasma membrane supported by an extensive network of endoplasmic reticulum (ER) and cytoskeleton material. Once the PPA is produced, local softening of the plant cell wall on the contact site is assumed to allow AM fungal hyphae to expand in the apoplastic tunnel (121).

Likewise, a similar structure called the infection thread (IT) develops when the rhizobia colonize root hairs in legumes (41). This reflects shared molecular and cellular mechanisms, which are orchestrated to a considerable extent by the CSP and defined by a set of genes that are indispensable for both nodulation and AM symbioses (122). The Symbiosis Receptor Kinase (LjSYMRK/MtDMI2; hereafter, SYMRK) (33, 157) resides on the plasma membrane and comprises an extracellular malectin (MLD) domain, a GDPC motif, and a leucine-rich repeat (LRR), as well as transmembrane and intracellular kinase domains (83). Ectopic expression of SYMRK causes interaction with both Nod-factor receptors NFR1 and NFR5, leading to spontaneous nodule formation and the induction of AM marker genes in the absence of microbes (138). In addition, the protein Remorin was proposed to serve as a scaffold for interaction among these receptors at a localized membrane subdomain (86). At the basal level, SYMRK undergoes rapid degradation by shedding its MLD domain (4), possibly through the action of E3 ligases known to interact with SYMRK (29, 188). This SYMRK cleavage is essential for its function, as demonstrated by the mutant allele *symrk-14*, which contains a point mutation in the GDPC motif and is therefore unable to be cleaved, resulting in defects at the epidermal penetration site (83). Interestingly, the cleaved form of SYMRK without its MLD preferentially interacts with NFR5 (4). Although the role of the increased interaction between SYMRK and NFR5 in symbiotic signaling is unknown, it further supports the notion that SYMRK functions as a coreceptor during symbiosis. Therefore, plants appear to modulate the degree of symbiosis development by adjusting SYMRK action via phosphorylation of the negative regulators plant U-box E3 ubiquitin ligases 1 (PUB1) (174) and 2 (PUB2) (91). PUB1 is also phosphorylated by NFR1 (MtLYK3) and has been suggested to limit overcolonization of the root by either endosymbiont (174).

Downstream of SYMRK, nuclear calcium spiking is central to signal transduction. However, it is not known how the plasma membrane–localized SYMRK activates calcium spiking in the nucleus. Based on the identities of SYMRK interacting proteins, it has been suggested that

mevalonate, a precursor of isoprenoid compounds, is the missing secondary messenger in the cytoplasm (74, 173). However, detection of mevalonate in the cytoplasm and evidence for nuclear-localized channel activation by mevalonate are required to prove that this is the case. Genetic screens have identified a number of signaling components in addition to the LysM-RLKs that are essential for calcium spiking, such as nuclear pore complexes NUP85, NUP133, and NENA (52, 73, 143); the nuclear envelope-localized calcium ATPase MCA8 (20); and the two K-channels, LjPollux (MtDMI1) and LjCastor (22, 65). More recently, three members of cyclic nucleotide-gated channel 15 (CNGC15) were identified as responsible for the release of calcium from the nuclear envelope lumen to the nucleoplasm (23). In the nucleoplasm, the calcium spiking activates the Calcium- and Calmodulin-dependent serine/threonine protein kinase (LjCCamK/MtDMI3) (87, 105) to phosphorylate the transcriptional activator LjCYCLOPS/MtIPD3 (63, 150), followed by downstream gene activation associated with host reprogramming (reviewed in 126).

In both legumes and rice, mutants with defects in CSP components exhibit similar phenotypes. Attempted colonization results in hyphopodia development, but the PPA does not form appropriately and thus rhizodermal penetration is limited (7, 45, 46, 54, 181). Supporting the role of CSP in PPA formation, ectopic overexpression of both SYMRK and constitutively active CCaMK variants induced symbiosis-specific genes in the absence of fungi, the latter also leading to the formation of a PPA-like structure (138, 162, 163).

Remarkably, however, knowledge on the spatiotemporal requirement for CSP components is scarce. Although it is well documented that CSP genes are not regulated in response to inoculation with AMF (54, 174), in which cell types these genes need to be expressed relative to the penetrating hyphae to enable AM fungal colonization has received little attention. In *M. truncatula ccamk* (*dmi3*) mutants, restoration of rhizodermal colonization was facilitated by introducing *CCaMK* under the control of a rhizodermis-specific promoter but not when using a cortex-specific promoter (139). Moreover, in *ccamk* mutants complemented with the rhizodermal construct, hyphal cortex colonization occurred but arbuscule formation was not observed. In analogous approaches studying nodulation in both *M. truncatula* and *L. japonicus*, rhizodermal expression of *CCaMK* or *CYCLOPS* was sufficient to rescue the rhizodermal mutant phenotype (60, 140). In contrast, using a rhizodermis-specific promoter to drive expression of *NUP85*, *NUP133*, *CASTOR*, or *POLLUX* in the respective mutant fully restored nodulation (60). Together, these data suggest that rhizodermal expression of signaling components upstream of the calcium spiking response is necessary and sufficient for nodulation (and possibly for AM symbiosis), whereas CCaMK and CYCLOPS are required throughout the infection process and, equivalently, for CCaMK in AM symbiosis (60, 140). Clearly, more work is required to map when and where, relative to the infection path of AMF, CSP gene activity is necessary to enable root colonization.

The functional relationship between D14L signaling and the CSP remains at present elusive. Lack of any AM fungal structures in *d14l* mutant roots and rhizodermal colonization of CSP mutants might indicate that D14L acts upstream of CSP. Alternatively, D14L signaling might operate in parallel to CSP, which is in agreement with early marker gene expression still occurring in CSP mutants of rice but absent from *D14L*-defective mutants (54, 56).

ARBUSCULE FORMATION

Following epidermal penetration, fungal hyphae proliferate inter- and intracellularly through the outer cortical layer via continuous formation of PPA (44). High-frequency calcium spiking was visualized as the fungi penetrated the cells, whereas low-frequency calcium spiking was observed in several neighboring cells where fungal hyphae subsequently entered via the PPA (149). Although

the molecules that trigger intracellular calcium spiking are unknown, it is possible that the AMF constantly produce GlcNAc signals.

Stage I: Inner Cortical Prepenetration Apparatus Formation

Upon reaching the inner cortex, AMF develop highly branched, tree-shaped feeding structures, the arbuscules (**Figure 3**). Unlike in the rhizodermis, PPA formation was accompanied by endoreduplication of the nucleus, likely reflecting the need for enhanced transcription in anticipation of accommodating the complex fungal structure (44). *L. japonicus cyclops* mutants failed to initiate arbuscule formation despite occasional intraradical proliferation of AMF (63, 125, 185), thereby demonstrating that CYCLOPS was indispensable for arbuscule development. Given the spatially broad *CYCLOPS* promoter activity in the root (104, 185), additional cotranscription factors might drive gene activation specifically to cells hosting arbuscules.

DELLA proteins have long been known as transcriptional repressors of gibberellic acid (GA) signaling that are degraded upon GA perception, whereby GA gene expression is enabled. In legumes and rice, *della* knockout mutants displayed severely diminished arbuscule formation (39, 40, 125, 162, 187), reminiscent of the *cyclops* mutant phenotype, which prompted examination of the genetic relationship between CYCLOPS and DELLA. Indeed, constitutive expression of a degradation-resistant version of DELLA complemented the *cyclops* mutant phenotype. Furthermore, direct interaction between DELLA and CYCLOPS was shown by both yeast–two hybrid assays and coimmunoprecipitation from tobacco leaves (39, 125, 162, 187). However, DELLA localized to the endodermis and vascular tissue (39), suggesting cell-to-cell movement to the inner cortex.

Stage II: Arbuscular Trunk Formation

A single fungal hypha progresses into the PPA of the inner cortex cell before extending dichotomously (**Figure 3**). Concomitant with the development of intracellular fungal structures, the hosting plant cell envelops the growing fungus with the periarbuscular membrane (PAM), which is continuous with the cell membrane. Although the mechanism underpinning dichotomous branching is unknown, live-cell imaging in *M. truncatula* demonstrated that the AM-induced Blue copper binding protein1 (BCP1) is localized to both the membrane surrounding the trunk domain and the plasma membrane but not the branch domain, thereby suggesting that the trunk domain retains properties of the plasma membrane (129).

Stage III: Arbuscular Basal Branching

From the trunk domain, AMF bifurcate to produce lower-order or basal branches. This appears to be a distinctive stage, as mutants of genes involved in fine branch formation, such as the AM-specific transcription factors belonging to the GAI, RGA, and SCR (GRAS) families, RAM1 and RAD1 (see below), still form the lower order of main branches (120, 125, 137, 183).

Stage IV: Arbuscular Fine Branching

By continuous dichotomous branching, the arbuscule further differentiates to develop higher-order fine branches until eventually filling the cortical cell. The size of the plant vacuole is reduced to accommodate the growing arbuscule, and the ER, peroxisome, Golgi apparatus, nucleus, and plastids are densely compacted within the cytoplasm (129). The PAM envelops the entire fungal

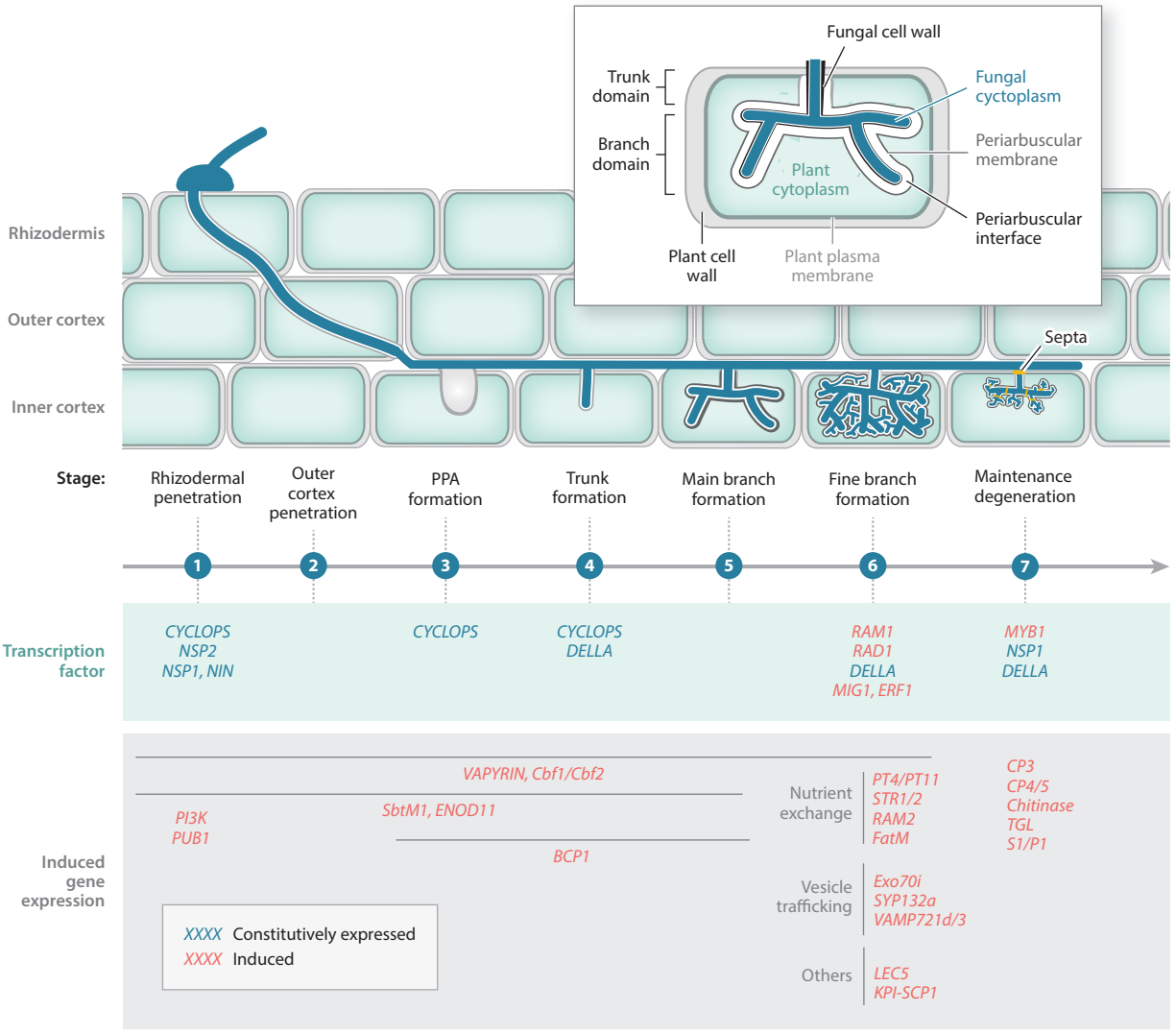

Figure 3

Transcriptional regulation of intraradical fungal colonization. Intracellular fungal colonization and arbuscule development are regulated by gene activation and governed by a network of transcription factors. ①–③ To achieve intracellular colonization, CYCLOPS (150) activates genes involved in signaling and vesicle trafficking to construct the prepenetration apparatus (PPA). ④,⑤ At the inner cortex layer, CYCLOPS interacts with DELLA to activate the major GRAS transcription factor ⑥ RAM1 to induce genes involved in fine branching, lipid biosynthesis, and nutrient exchange (39, 47, 97, 120, 125, 137, 183, 187). Proper targeting of nutrient transporters occurs via polarized exocytosis to the periarbuscular membrane surrounding newly developing fungal branches (64, 67, 96, 119, 130, 191). Simultaneously, RAM1 and DELLA interact with other transcription factors such as RAD1 and DIP1 (120, 183, 187) to maximize gene expression while MIG1 contributes to the radial expansion of arbuscule colonization (61). ⑦ The arbuscule degenerates, involving the MYB1-NSP1-DELLA transcription factor complex to induce hydrolases (38). Abbreviation: PPA, prepenetration apparatus.

structure, thereby creating a large membrane surface area and periarbuscular interface (PAI) for the exchange of nutrients and signals between the two organisms (**Figure 3**).

Plant Pi transporters mediating symbiotic Pi uptake localize to the PAM fine branch domain (59, 69, 79, 184). Considering the continuum of plasma membrane domain to trunk domain to basal and fine branch domain, such polar localization is indeed remarkable. Promoter swapping experiments proposed that the timing of expression determined the protein export route to the PAM subdomain surrounding the growing fine branch (130). Polarized secretion is then mediated by exocytosis, including the focalized fusion of membrane vesicles. Loss of function mutations of a subunit of the exocyst complex *Exo70i*, which might be responsible for tethering of secretory vesicles to PAM, produced as many arbuscules as wild type; however, the higher-order fine branches were never observed (191). Strikingly, the precise focal localization of *M. truncatula* Exo70i in juxtaposition to the PAM subdomains opposite hyphal tips suggested a distinct role in polar secretion, possibly increasing the magnitude of exocytotic activity to support the development of the arbuscule (191). Similarly, mutants of SNARE proteins involved in vesicle fusion to the PAM such as *SYNTAXIN 132a*, *VTI12*, and *VAMP72d/e* were also defective in arbuscule development (64, 67, 96, 119). Collectively, these reports revealed that directed exocytotic activity toward fine branches is critical for arbuscule development and symbiosis maintenance, plausibly for delivering building blocks for PAM biogenesis. However, the signal(s) that promotes secretion to PAM remains to be discovered.

To coordinate the remodeling of the cell architecture supporting this extraordinary structure, the arbuscule-hosting cell undergoes extensive transcriptional reprogramming. In anticipation of the increasing arbuscule volume during fine branch formation, GRAS transcription factors MIG1 and DELLA execute radial cell expansion (61). More importantly, forward genetic screens and transcriptomic analysis of arbusculated cells combined with reverse genetics identified a GRAS transcription factor, Reduced Arbuscular Mycorrhiza1 (RAM1), as essential for intracellular colonization and specific for AM symbiosis (47, 120, 125, 137, 183). Legume and petunia *ram1* knockout mutants exhibited reduced overall intraradical fungal proliferation; once they formed arbuscules, they produced severely underdeveloped fine branches. Interestingly, *RAM1* promoter activity occurred in all cell layers of colonized root segments (120, 125, 183). If the RAM1 protein was present in the same tissue domain as the promoter activity, achieving specific gene expression in arbusculated cells would thus require another transcription factor. However, although several transcription factors, such as RAD1 (120, 183) and DIP1 (187), were interacting with RAM1, evidence for cortex cell–specific expression has not yet been reported. An elegant study recently demonstrated that the phosphorylated CYCLOPS-DELLA complex activated the *RAM1* promoter to promote AM-specific gene expression (125), raising the question of how AM signaling, relative to nodulation signaling downstream of CYCLOPS, is achieved.

Stage V: Arbuscule Senescence

Remarkably, despite the monumental cellular efforts to produce and accommodate arbuscules, they are ephemeral structures with a life span of a few days. The mechanisms that control cellular dynamics associated with arbuscule senescence are not known. According to microscopic documentation, hyphal collapse is initiated from the fine branches and gradually progresses toward the trunk, preceded by the retraction of the fungal cytoplasm and accompanied by an increased production of septa (2). Using vital staining, it was found that the trunk retained vitality even when the arbuscule branch domain underwent senescence (78), which has been attributed to the development of distinct septa at the base of the trunk to separate the degenerating hypha from the living hypha.

The recent identification of the first transcription factor, MYB1, controlling arbuscule degeneration represents a major breakthrough (38). In *M. truncatula* mutants deficient for the symbiotic Pi transporter *PT4*, arbuscules exhibited an accelerated turnover (69) that was associated with the launching of a transcriptional program enriched for genes encoding hydrolytic enzymes, indicative of an active contribution of the plant cell to the digestion of the collapsing arbuscule (38). The MYB1 transcription factor had increased transcript levels in arbusculated cells and regulated the expression of the hydrolytic genes responsible for degeneration of the arbuscule, as evidenced by the restoration of the arbuscule life span in *pt4/myb1* double mutants (38). Therefore, it is possible that an imbalance in the reciprocity of nutrient exchange is introduced between the partners because *pt4* mutants are unable to obtain Pi from the fungus, which leads the plant to degrade the invader. In this model, fungus-delivered Pi functions as an important signal in the arbusculated cell for the suppression of the host's digestive machinery. Somewhat surprisingly, MYB1 functioned in conjunction with transcription factors DELLA and NSP1 (38), both of which play a role in the development of symbiosis (26, 39, 125, 164, 187) and have been newly assigned to participate in the regulation of arbuscule degradation. Although experimentally not examined, it is conceivable that during the consecutive developmental stages of the arbuscule life, distinct signaling pathways target the activation of these GRAS transcription factors, resulting in profoundly different outcomes.

Considering the vast amounts of arbuscular, PAM, and interface material, it is intriguing to speculate about the further fate and role of the disassembled constituents. Time-lapse images of rice roots documented that the fungal lumen in the main branch and trunk domain accumulated lipids as the arbuscules degenerated (78). Although arbuscule degeneration potentially has an important role in carbon and Pi homeostasis for both symbionts, whether or not it contributes to nutrient transfer between the symbionts requires further investigation.

VESICLE AND SPORE FORMATION

Upon the successful establishment of AM symbioses, AMF produce lipid storage vesicles, which are thick-walled, irregular lobes that are intraradically formed either within or between host cells by most orders of Glomeromycotina fungi except the Gigasporales (151). Similarly, appropriate nourishment of the fungus by the plant leads to the production of asexual spores that in addition to nuclei also contain storage lipids in the form of triacylglycerol (TAG) to support spore germination and asymbiotic germ-tube growth (102). Although it is unknown how vesicle and spore formation are regulated, correlative evidence linked vesicle formation with lipid transfer from the host, as demonstrated by the reduced vesicle formation in lipid metabolism mutants such as *dis*, *ram2*, and *str* (see section below) (75; reviewed in 136, 142, 177).

BIDIRECTIONAL NUTRIENT EXCHANGE

Reciprocal nutrient exchange between plant and fungus lies at the heart of mutualism in AM symbioses (**Figure 4**). AMF deliver Pi and nitrogen to the plant in exchange for organic carbon. Using high-affinity Pi transporters, the extraradical mycelium of AMF acquires inorganic Pi from the soil (59) and converts it into polyphosphates for storage and transport from the extraradical to the intraradical mycelium within the vacuolar compartment (154). Inside the fine arbuscular branches, hydrolysis of polyphosphates and subsequent release of inorganic Pi into the PAI allow phosphate to be available to plants that use distinct PAM-intrinsic Pi transporters for the rapid uptake into the plant cortical cells (58, 69, 79, 124, 133, 184). The plant's Pi status is critical for symbiosis establishment and maintenance. Plants regulate the symbiosis in response to Pi levels

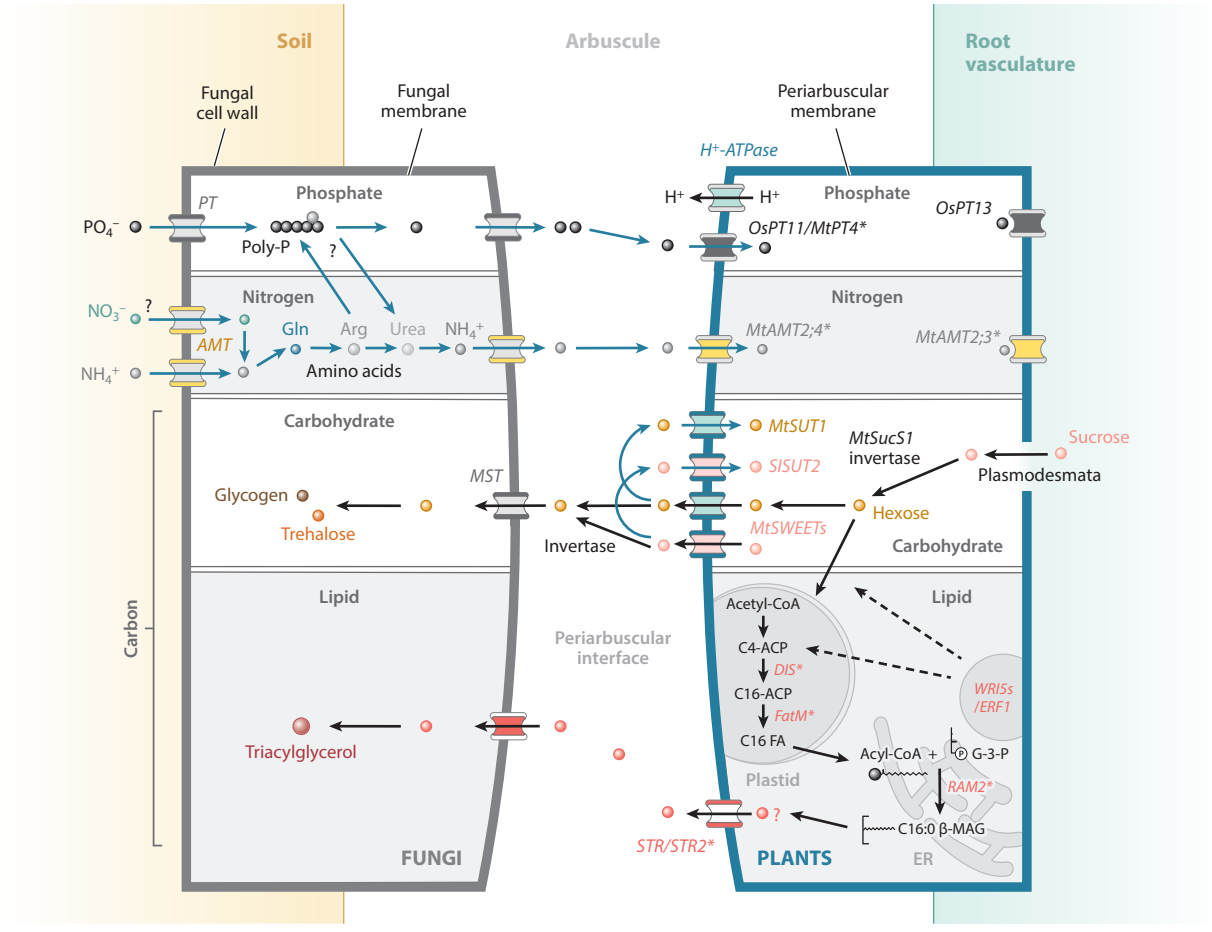

Figure 4

Bidirectional nutrient exchange. The hallmark of the arbuscular mycorrhizal (AM) symbiosis is the reciprocal exchange of nutrients. AM fungi (AMF) provide soil-acquired phosphate and nitrogen in exchange for organic carbon. Fungal polyphosphates are transported to the arbuscules, where they are hydrolyzed into the inorganic form to be unloaded into the periarbuscular interface (PAI) (154). From here, symbiosis-specific phosphate transporters, belonging to the MtPT4/OsPT11 type, acquire the phosphate across the periarbuscular membrane (PAM) (58, 69, 79, 124, 133, 184). Similarly, soil-acquired nitrogen arrives in the arbuscule in the form of amino acids (glutamate and arginine), where, after conversion into urea and ammonia, the latter is released into the PAI (50). Plant ammonium transporters, possibly of the MtAMT2;4 kind, take up ammonium across the PAM (15). During AM symbiosis, the roots gain sink strength to provide photosynthates to AMF in two forms: sugars and lipids. In the PAI, sucrose is broken down into hexose, with glucose being predominantly acquired by fungi and converted into the storage forms trehalose and glycogen (62, 148; for complementary, detailed reviews, see 136, 142, 147, 177). AMF lack genes for long-chain fatty acid (FA) biosynthesis (72, 182) and therefore depend on the host for their supply. De novo FA synthesis occurs in arbusculated cells by expressing the FA biosynthetic genes *WRI5b/EFR1* (transcription factor), *DIS* (FA synthase), *FatM* (acyl transferase), and *RAM2* (GPAT6), and PAM-localized lipid transporter candidates *STR/STR2* (13, 70, 75, 97). The provided long-chain FAs are converted into triacylglycerol (TAG) for storage or, alternatively, desaturated for use in growth and development. The major transcription factor RAM1 activates genes involved in nutrient exchanges (*asterisk*). Abbreviation: ER, endoplasmic reticulum.

using hormonal signaling. Under Pi deficiency, the resulting increased release of SLs facilitated AM colonization (84, 94). However, high levels of Pi stimulated expression of GA biosynthetic genes, leading to DELLA degradation and subsequent reduction of arbuscule development (39). Similarly, AMF contribute to plant nitrogen nutrition by the transfer of soil-acquired inorganic forms of nitrogen to the host plant (50). In *M. truncatula*, ammonium transporter *AMT2;3* was induced during arbuscule development and, remarkably, required to suppress premature arbuscule degeneration in the *pt4* mutant under low-nitrogen conditions (15). As introduction of *AMT2;3* did not complement the yeast ammonium uptake mutant, the authors proposed that AMT2;3 may function as a transceptor involved in sensing rather than in transporting ammonium (15). Together, the host cell appears to carefully monitor the fungal delivery of Pi and nitrogen to continuously support the maintenance of the AM symbiosis, thereby reinforcing the dual role of Pi and (likely) ammonium as nutrients and signals.

AMF are obligate biotrophs that depend on their host for carbon supply to complete their life cycle, which is reflected by the formation of daughter spores. Utilizing C^{13}-radioisotope labeling, it has been suggested that the main form of carbon delivered to fungi is glucose (148). This glucose is taken up by a high-affinity fungal monosaccharide transporter and then converted into glycogen and trehalose for export through the rest of the fungal mycelia (62, 147; for complementary, detailed reviews, see 136, 142, 177).

Considering that the majority of fungal hyphae, vesicles, and spores are packed with lipids, it has been proposed that carbohydrates are converted into lipids (167). Recently, however, advances in fungal genomics (141, 144, 165, 166) revealed that AMF lacked eukaryotic FA synthase (FAS) genes that are required for the de novo synthesis of long-chain FA, implying a dependence on their hosts for FA supply (72, 182). Consistently, the expression of both fungal and host genes involved in lipid metabolism and transport were elevated in arbuscule-containing cells (14, 28, 42, 48, 182). In a spectacular parallel effort, four groups recently reported a linear pathway for the de novo FA synthesis in plants that are associated with carbon nourishment of the fungus (13, 70, 75, 97). The AM-specific RAM1 transcription factor was required for induction of a gene encoding the α-keto-acyl ACP synthase (KAS) III, which is involved in the early stages of FA biosynthesis by elongating C2:0-ACP to C4:0-ACP (75), suggesting that the host activated de novo FA synthesis. C4:0-ACP is then further extended into C16:0-ACP by disorganized arbuscules (DIS), encoded by the *KASI* gene (75). At this point, AM-specific acyl thioesterase, FatM, releases C16:0 FA from acyl carrier protein (ACP) for subsequent export to the cytosol, where C16:0 FA is conjugated to CoA to be transported to the ER. *RAM2* encodes a glycerol-3-phosphate acyl transferase (GPAT) and attaches C16:0-FA to the second carbon of glycerol-3-phosphate to make C16:0 monoacylglycerol (C16:0 β-MAG). Although direct transport activity has not been shown, lipid profiling strongly supports the idea that C16:0 β-MAG or further processed lipids are exported to the PAI by a heterodimer complex of the half-size ATP binding cassette transporter subfamily G (ABCG) STR/STR2 (13, 70, 75). The presence of genes involved in FA desaturation and elongation in fungal genomes supports the hypothesis that AMF can utilize plant lipids for growth and development once long-chain FAs are provided from the host (72, 182). Evidence for FA provision by the host was obtained by fungal lipid analysis after cocultivation of *R. irregularis* with plants that were supplemented with radiolabeled carbon nutrients (75, 97). Furthermore, metabolically engineering *M. truncatula* to produce C12:0 FAs, which are otherwise rarely detected in hosts and fungi, showed that fungal TAG indeed contained C12:0 FA moieties when in association with roots producing C12:0 FAs, thereby demonstrating that the host provided FA groups to the fungi (70, 97). Consequently, plant mutants involved in FA synthesis and transport have equivalently reduced fungal colonization, indicative of the malnourishment of the fungus (13,

14, 31, 51, 57, 70, 75, 97, 176, 189) and its inability to produce vesicles and spores (75) and thus to complete the asexual life cycle (142).

Interestingly, asexual spore formation in another early diverging fungus, *Rhizopus microsporus*, a member of the Mucuromycotina, an early divergent sister clade of the Glomeromycotina, also depended on the accumulation of lipids, namely TAG and phosphatidylethanolamine (PE) (85). Here, equimolar ratios of TAG/PE are required for the mutualistic interaction with β-proteobacteria *Burkholderia*, explaining the dependence of *R. microsporus* on the bacterial endosymbiont for asexual reproduction. Similarly, fungal-bacterial mutualism has been reported between the AMF species *Gigaspora margarita* and *Candidatus* Glomeribacter gigasporarum, an endobacterium closely related to *Burkholderia*, which also promoted fungal sporulation (144). Still, although this association improved presymbiotic fungal performance and asexual reproduction, it remains to be seen whether lipid metabolism plays a critical role in this relationship.

OUTLOOK

Recent findings have shed valuable light on the molecular processes underlying the dynamic establishment of AM symbioses. Still, to truly grasp the way this intimate symbiosis is formed, several points require particular attention. Although the characterization of Myc-LCOs and $CO_{4\text{-}5}$ have opened up paths for exploration, our understanding of the plant-fungal rhizosphere dialog has notable gaps. The discoveries of NOPE1 and D14L add important new players to presymbiotic signaling; identifying the bioactive molecules they transport or bind would enable a much deeper understanding of how plant and fungi perceive mutual presence. In addition, although D14L and NOPE1 are conserved throughout land plants, their symbiotic roles beyond monocots also need to be addressed. Likewise, our understanding of how nutrient exchange is regulated once the arbuscule is formed, in particular the mechanisms that control reciprocal mineral and carbon delivery, is still unclear. Identifying FAs as the major form of plant organic carbon for fungal nourishment has been a game-changing finding. Building from here, the mode of FA delivery, its spatiotemporal coordination during arbuscule development and senescence, relative to phosphate uptake, invite further studies. The process of arbuscule senescence also raises the intriguing question of the mechanistic regulation behind this stage and its degree of importance for the maintenance of AM symbiosis. Then, finally, although the formation of vesicles and spores is often viewed as an indicator of fungal fitness, the molecular mechanisms that regulate formation are still not well understood. Examination of these points, among others, will open new avenues of research. This will build an increasingly clear picture of the underpinnings for development and maintenance of this prevalent, mutualistic plant-fungal symbiosis.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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