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(Article begins on next page)


**Dating in the dark: how roots respond to fungal signals to establish arbuscular mycorrhizal symbiosis**

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**Abstract**

The arbuscular mycorrhizal symbiosis that involves most plants and Glomeromycota fungi is the result of a complex exchange of molecular information, which commences before the partners are in physical contact. On the one hand, plants release soluble factors, including strigolactones that activate both the metabolism and branching of the fungal partners. On the other hand, fungi use compounds that trigger the signaling transduction pathways that are required for the symbiotic modus of plant cells. Here we describe some of the recent discoveries regarding the fungal molecules involved in rhizospheric conversation, and the way in which they are perceived by their hosts. We conclude that similar signaling molecules may have different meanings, depending on the context. However, at the end, specificity must be maintained to ensure appropriate partners enter symbiosis.

**Introduction**

As sessile organisms, plants are exposed to a dramatic variety of beneficial microbes and pathogens from all kingdoms. In order to deal with the latter, plants have developed an innate immune system, which employs complex mechanisms based on specific signaling, hormonal changes, and transcriptional reprogramming [1]. However, resistance to pathogens implies costs and plants have to quickly identify benign organisms to avoid defense elicitation. Arbuscular mycorrhizal fungi (AMFs), soil inhabitants of the ancient Phylum Glomeromycota, establish mutualistic interactions with roots of most terrestrial plants [2]. AMFs play a major role in nutrient cycling and improve the nutrient status of their host, influencing growth, water absorption, and protection from root diseases [2]. Consequently, the plant mechanisms used to recognize and establish molecular dialog with AMFs which eventually culminates in symbiosis are of crucial importance in plant biology. Many important aspects concerning the molecules released by the plants and their multiple roles have been dealt with in recent reviews [3 and 4]. Therefore, we here focus on the bioactive molecules released in the rhizosphere by AMFs, as well as on the molecular mechanisms of their perception by the plant.

**Soluble fungal signals in the rhizosphere**

For obligate biotrophic microbes such as AMFs it is mandatory to maximize the chance of encountering a host before depletion of the spore resources. The release of soluble signals in the rhizosphere is thus an easy solution to allow both partners to be timely informed of their presence, even before physical contact [5]. The pioneering work of Kosuta et al. [6] postulated the existence of an AMF diffusible signal that induced the expression of the plant gene *MtEnod11*. Other studies have demonstrated that such molecules have an extensive effect on genes and signal transduction processes [7, 8 and 9], root branching [10 and 11], and sugar metabolism [12]. These presymbiotic responses have been described as the ‘anticipation program’, which prepares the symbionts for a successful association [13].
Myc factor: no longer the Holy Grail? In analogy with rhizobial Nod factors, the AMF diffusible molecules that assist the plant in recognizing them as friends and not as foes were named Myc factors. However, it was unclear whether the secretion of Myc factors was induced after the perception of plant signals or, alternatively, they were constitutive, allowing AMFs to be always ready for root colonization. The chemical nature of the Myc factor has been considered a sort of Holy Grail for many years, even though AMF bioactive molecules have been suspected of possessing a chitin backbone [14]. The long-awaited structure of at least one Myc factor has recently been disclosed: active factors are lipochitooligosaccharides (Myc-LCOs) which are very similar to Nod factors [15]. On the basis of the idea that the Myc factor molecule could be an ancestral form of the more modern Nod factor, the group headed by J Denarie set up a chemical approach to purify molecules based on their activity in bioassays designed for Nod factor detection: the Vicia sativa root-hair branching assay, the MtENOD11 activation test, and the M. truncatula root branching assay [15]. By mass spectrometry, the active molecules either from root organ cultures colonized by Glomus intraradices or from germinated spore exudates were characterized as tetrameric chitooligosaccharides, N-acylated with a C16 or C18 fatty acid moiety either saturated or having one or two unsaturations (Figure 1). Such Myc-LCOs may or may not be decorated by a sulphate group. Since fungal molecules are only produced in picogram quantities, their biological activities have been studied using synthetic Myc-LCOs obtained via bacterial genetic engineering [15••]. Myc-LCOs were shown to stimulate AM formation in legume and non-legume plants, to slightly induce the transcription of some AM-responsive genes, and to promote root branching in several plants. Interestingly, Nod factors had previously been described as good mycorrhizal inducers [16]. This suggests an overlapping function of the Myc and Nod Factor and opens the question concerning whether the reverse is also true: Are Myc-LCOs nodulation stimulants?
Two main groups of bioactive molecules produced by the plant and the fungal partner have been identified so far as being crucial in initiating the molecular dialog in arbuscular mycorrhiza: (a) The strigolactone group [48] carotenoid-derived metabolites produced by many plants including the AM host plant *Lotus japonicus*; (b) lipochitooligosaccharides [15••] produced by *G. intraradices* grown in axenic conditions in root organ cultures.

The discovery nicely supports the *tinkering* concept that Jacob [17] used to explain how organisms use available elements during evolution to invent novel functions according to their needs. In this way, very similar molecules may guide two morphogenetically and functionally diverse plant–symbiotic interactions: the widespread 450My old AM symbiosis and the legume association with the younger 60My old rhizobial interaction. At the same time, the Myc-LCOs discovery leads to several important questions, concerning for instance the mechanisms by which a huge number of bacteria, from Rhizobia to Burkholderiaceae, have acquired the capacity to synthesize a molecule of fungal origin: by gene horizontal transfer or by an independent synthetic pathway? The availability of the *G. intraradices* genome will be crucial in the identification of fungal genes involved in the synthesis of Myc-LCOs and in understanding whether the perception of plant signals is a pre-requisite for their activation.

### On how plants detect AMFs

Plant perception of fungal diffusible signals is translated in a transcriptional response that initiates and prepares the plant for fungal accommodation. For more than 10 years now, it has been known that the process of AM formation and nodule symbiosis shares a partially overlapping signal transduction route, the common symbiosis (SYM) pathway [18]. In this route, microbial signals are interpreted into a calcium signal that determines the activation of essential symbiotic genes [19]. However, the initial and last stages of the SYM pathway differ for bacterial and fungal symbionts. In the two model legumes, *Lotus japonicus* and *M. truncatula*, the putative receptors for Nod factors have been identified (NFP and LYK3 in *M. truncatula*; NFR1 and NFR2 in *L. japonicus*) and positioned at the top of the SYM pathway. These receptors are membrane kinases that contain LysM motifs in their extracellular domains [20, 21 and 22]. Mutant plants defective in these receptors are still capable of mycorrhiza formation [23], and their epidermal cells still respond to mycorrhizal signals and induce calcium oscillations, for example, in *M. truncatula* plants lacking NFP [24•]. Therefore, it has been assumed that Myc factors are perceived through different receptors. Similarly, three transcription factors are specifically activated upon the perception of Nod factors (NSP1, NSP2, and ERN), but their deletion does not affect AM formation [18]. However, this information should be re-evaluated in light of the results of Maillet et al. [15••], who suggest that both, the NFR receptor and the NSP2 transcription factor are involved in the perception of Myc-LCOs (Figure 2).
Figure 2.

The scheme illustrates the signaling transduction pathway in Rhizobial and mycorrhizal symbiosis. While Myc-LCOs are expected to travel along the same pathway required for nodule formation, the so-called common symbiosis (SYM) pathway, additional routes for additional AM signals are expected. Thus, other Myc factor signals (i.e. COs) may also function in a SYM-dependent manner, while others, whose nature is still unknown, are SYM independent.

These observations lead to important questions. On the one hand, it is expected that the Myc-LCO receptor will also be an LysM-containing protein. However, most Rhizobia have a narrow host range determined by the chemical structure of the Nod factor. In contrast, AMFs are very promiscuous: one fungus can infect several plants while one plant can be colonized by several AMFs. In addition, G. intraradices produces a mixture of four lipochitooligosaccharides [15]. Are these the same as those produced by other AMFs? And, does a plant need several Myc factor receptors to recognize all the AMFs? Or, are all Myc factors perceived by one and the same receptor? Alternatively, there could be additional mycorrhizal produced LCOs or alternate signals yet to be defined.
One possible answer might be that some broad spectrum rhizobial strains are capable of infecting more than one legume species [25]. They produce simpler Nod factors with low unsaturated fatty acids comparable to those of G. intraradices. It is believed that the narrow host range of Rhizobia is actually a specialization that has evolved because of modifications of these simpler Nod factor forms with three to five chitin residues N-acylated with a C18 fatty acid [25]. Another indication of this is the recent finding of the NFP ortholog in Parasponia, the only non-legume plant able to engage in rhizobial symbiosis [26]. NFP silencing in Parasponia impairs symbiosis formation with both rhizobia and AMFs, which would seem to suggest that this receptor is required for both associations. If this is the case, why do NFP legume mutant plants still respond to AMF with calcium spiking? A mechanism which guarantees that perception of the Myc factor is not confused with other similar rhizospheric signals, such as Nod factors, must surely exist. It is likely that other, as yet unknown, mycorrhiza-specific receptors might control the specificity of the downstream cascade in response to other different fungal signals from Myc-LCOs (Figure 2).

How are symbiotic signals transduced?

Calcium is the most widespread intracellular messenger. It couples a wide array of extracellular stimuli to specific physiological responses [27]. In analogy with nodules, where Ca2+ oscillations are considered the landmark of a successful interaction [28], Ca2+ has long been hypothesized to be involved in AMF signal transduction [29]. However, the first experimental evidence of Ca2+ as a transducer of mycorrhizal signals came from measurements of Ca2+ oscillations in M. truncatula root hairs in the presence of, but not in contact with AM hyphae [30]. The use of a nuclear cameleon probe has provided two additional crucial contributions: firstly, the nucleus is the main target of the signaling pathway; secondly, the nuclear location of Ca2+ oscillations is an indispensable step for the activation of the nuclear kinase DMI3 [24]. AMF exudates, as well as hyphopodium contact, are able to induce Ca2+ oscillations in the epidermal cells of legume and non-legume plants (Figure 3). Furthermore, the root regions of that responsive to AM signals were located between 1 and 2 cm from the tip of young lateral roots [24]. Like the rhizobium–legume symbiosis, Ca2+ oscillations were detected in dmi3 but not in dmi1 and dmi2 Medicago mutants in response to AMFs that support the hierarchical distribution of the SYM pathway with the nuclear kinase DMI3 located downstream as the decoder of the calcium spiking [24 and 30]. On the one hand, it seems evolutionary convenient that the same subset of genes, which are common in plant taxa [31] and have been shown to function in mycorrhizal signaling in non-legumes [32], could control two diverse biological systems. On the other hand, this common response again raises the problem of specificity: how do legumes distinguish between AMFs and rhizobia and organize the appropriate accommodation of the microbe inside the root tissues? The answer is that plant cells can possibly originate different calcium signatures in response to different stimuli by changing the duration of the Ca2+ concentration peaks as well as their frequency [33]. In fact, AMF-related nuclear calcium spikes are less frequent and more irregular than those induced by Nod factors [24, 30, 34 and 35]. Computational analyses have suggested that both Myc and Nod-stimulated Ca2+ oscillations are chaotic in nature. This might explain the flexibility with which the SYM pathway produces and interprets different calcium patterns, since chaotic systems are very flexible and sensitive to small changes in the nature of the input and have minimal energetic requirement [34].
Figure 3.

The scheme illustrates the method used to investigate calcium spiking in response to fungal exudates produced by germinating spores of *Gigaspora margarita* applied to root organ cultures (ROC). Roots expressing a nucleoplasmin (Nup) cameleon Ca^{2+} sensor construct were analyzed and the nuclear oscillations were recorded as spiking [24].

The type of calcium signal produced by the newly identified Myc-LCOs is still not known (Figure 2). However, on the basis of their chemical structure it can be expected that the oscillations are similar to those produced by simple Nod factors. Interestingly, Nod factors do not induce calcium spiking in the epidermal cells of root organ cultures while AMF exudates [24] and short-chain
chitooligosaccharides (A Genre et al., unpublished data) still do. This would seem to suggest that calcium spiking during AMF perception might be shaped by more than one AMF signal (Figure 2). The root organ culture system will allow one to investigate the ability or lack of ability of Myc-LCOs to induce calcium spiking and perhaps to discriminate between different fungal signals.

It is generally assumed that CCaMK (DMI3) is the essential decoder of the calcium signal. Gain-of-function mutations of the corresponding gene eliminate the need of the upstream SYM component for both nodulation and mycorrhization [36 and 37]. Interestingly, dominant forms of this kinase induce nodulation in the absence of bacteria or Nod factors [38]. Therefore, why has spontaneous nodulation never been observed in non-legume plants, where DMI3 is also present, in response to Myc-LCOs? A likely explanation is that different specific mycorrhizal signals from Myc-LCOs could act as negative regulators of the ‘default’ nodulation pathway that travels along the SYM pathway. An alternative explanation is that essential nodulation components downstream of CCaMK are absent in non-legumes.

**Conclusions and perspectives**

It is now clear that crucial signaling events for mycorrhizal symbiosis occur in the rhizosphere, before plants and AMFs come into contact, through an exchange of diffusible compounds. A plethora of signal molecules, including those from other inhabitants of the soil are expected to be present. Interestingly, the chemical nature of the mycorrhizal symbiotic molecules identified so far points to their flexibility as signals: plants constitutively release the versatile strigolactones, which also act as germination inducers of parasitic plants [3and4] as well as hormones that are responsible for plant architecture [39]; and the active molecule produced by the AMFs mirrors the Nod factor released by the rhizobia. It seems that in the rhizospheric conversation, the same word might have different meanings depending on the context. However, in the end, only the two appropriate partners end up living in symbiosis.

At more advanced stages, when physical contact occurs at the root epidermis and developmental processes such as hyphopodia formation and epidermal re-organization take place [5] the signal production/perception picture will certainly become more complex (Figure 4). Other molecules, including those already identified (i.e. phosphate, lysophosphatidylcholine, hormones, and microRNAs), are likely to be involved at this stage and are probably responsible for the responses caused by AMFs at a plant systemic level [40, 41, 42 and 43]. Plant mutants affected in the later stages of the symbiosis will help one to dissect which signals are primary or secondary in the accommodation process within the root [44]. With this knowledge in hand, it will also be possible to learn more about the commonalities with and differences from other plant–microbe interactions, including why similar signals lead to differential morphogenetic processes, as in the case of Myc-LCOs and Nod-LCOs, and how AMF have learned to escape the default fungal recognition program of the plant. The recent finding that R genes restrict host specificity in N2-fixing symbiosis [45] suggests that effector proteins are required, as in pathogenic interactions, to evade the plant immune defense response and to establish the nodule symbiosis. This opens the compelling question concerning whether AMFs also make symbiotic use of pathogenic strategies as rhizobia [46]. Novel results in fact indicate that this might be the case and that AMFs possess and deliver effector molecules to counteract initial plant defenses (K Kloppholz et al., unpublished data). Alternatively, and because of their ancient origin and long-coevolutionary history with plants [47], it is also possible that AM associations may have predated the development of the plant innate immune system.
Figure 4.

Schematic summary of the steps that characterize the molecular dialog between AMFs and their host plants. (a) Both partners release bioactive molecules in the rhizosphere, as shown in Figure 1; (b) after perception of the signal by the reciprocal partner, specific molecular, cellular, and morphogenetic responses are elicited; (c) after hyphopodium formation, a successful colonization process is ready to start.

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