Signalling in Ectomycorrhizal Symbiosis

Judith Felten, Francis Martin, and Valérie Legué

Abstract The mechanism by which tree roots and soil fungi interact and form their common, symbiotic organ, the ectomycorrhiza (ECM), involves numerous steps. During this ontogenic process, the developmental programs of both partners are modified in order to enable symbiosis establishment. Both roots and fungus release an array of various metabolites (morphogens and signalling molecules) that establish a molecular cross-talk between symbionts. In contrast to some other plant–microbe interactions, such as rhizobia or arbuscular mycorrhiza symbiosis, the characterization of these signalling molecules and their impact on developmental pathways is poorly known. Recent studies have provided new insights into specific phases and signalling pathways of ECM development on a molecular level and have thereby started to fill the gaps in our understanding of root–fungus communication. Based on this knowledge and recent data from ECM interaction, we will identify possible crosstalk between ECM signalling and root development.

1 Structure and Development of Ectomycorrhizae

While only around 3% of seed-bearing plants establish an ectomycorrhizal (ECM) symbiosis with fungi, ECMs are the most common form of symbiosis for the majority of boreal and temperate forest trees. The fungal community involved in

J. Felten
UMR 1136 Interactions Arbres Micro-organismes, Centre INRA de Nancy,
54280 Champenoux, France

Department of Forest Genetics and Plant Physiology, Umeå Plant Science Centre, Swedish
University of Agricultural Sciences, 90 187 Umeå, Sweden

F. Martin • V. Legué (✉)
UMR 1136 Interactions Arbres Micro-organismes, Centre INRA de Nancy,
54280 Champenoux, France
e-mail: fmartin@nancy.inra.fr; Valerie.Legue@scbiol.uhp-nancy.fr

ECM formation concerns a great diversity of species among Basidiomycota and Ascomycotina fungi (Smith and Read 2008). Plants species forming ECM belong to Pinophyta and Angiosperms in temporate, boreal, and partially tropical forest (Smith and Read 2008).

The crucial impact of ECM fungi on tree nutrient uptake, including uptake of major nutrients such as nitrogen (N), phosphorus (P), and magnesium (Mg), is well established (reviews Chalot et al 2006; Martin and Nehls 2009). In parallel, the change in tree carbon (C) allocation caused by ECM fungi is considerable. These metabolism activities confer a major role of ECM in ecological and biogeochemical processes in forests (Taylor 2002; Buée et al. 2009).

The functioning structure defined as an ECM is characterized by a basic pattern including the presence of a typical mantle of fungal hyphae around the root, and a labyrinthine inward growth of hyphae between epidermal and (in some species) cortical cells, called the Hartig net (Blasius et al. 1986; Fig. 1a), while the intracellular penetration is scarce. An outwardly growing network of hyphal elements, the extramatrical mycelium, is seasonal present. This well-conserved feature drives the nutrient flow between the two partners (Martin 2007). Extramatrical hyphae gather nutrients (mostly N and P) from the soil and transport them to mantle hyphae, where they may be stored. From there, nutrients are transported to Hartig net hyphae and delivered to and taken up by root epidermis (and cortex) cells. Vice versa,
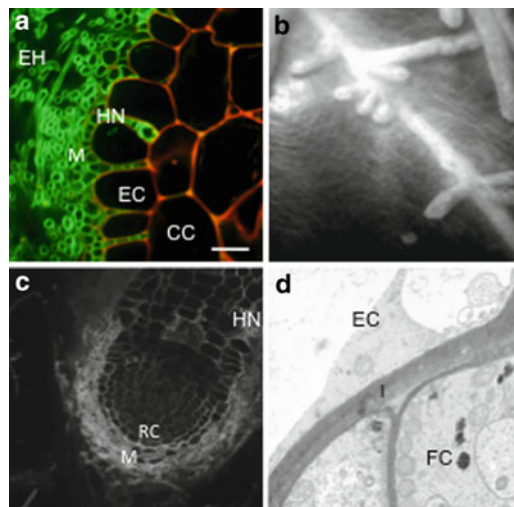

Fig. 1 Anatomy and structure of ectomycorrhizae. (**a**) *Populus tremula x Populus alba/Laccaria bicolor* root cross section. Fluorescent double staining showing peripheric root cells (*red cell wall*) and fungal hyphae (*green cell wall*). Mantle (M), Hartig Net (HN), extramatrical hyphae (EH), root epidermis cells (EC), root cortex cells (CC). Bar 10 μm. (**b**) Ectomycorrhizal *Poplar/Laccaria bicolor* short roots. (**c**) Poplar root apex colonized by *Laccaria bicolor*. The mantle (M) is present in the root apex, but the Hartig net is developed behind the root meristem. The ECM is accompanied with a reduced root cap (RC) and a small apical meristem zone. Bar 20 μm. (**d**) Interface (I) between root cells (RC) and fungal cells (FC) inside the Hartig net. Courtesy J. Gérard (Nancy University)

photoassimilates (sugars) are released by plant root cells and taken up by the fungus before (partially) being transported back into extramatrical hyphae that use them for further growth and fruiting body development. Together, the different structures and nutrient flow stimulate the growth and health of the plant on several levels: (1) thanks to the wide network of extramatrical hyphae, it benefits from nutrient resources from a greater soil volume than it could exploit by its roots alone (Rousseau et al. 1994), (2) the secreted enzyme activities, which increase mineral solubilization in the soil, can enhance nutrient absorption by plant roots (Landeweert et al. 2001), and (3) the enclosure of the root by the fungus protects plant roots against pathogens but also against inorganic agents. In specific cases, the ability of fungi to absorb and store heavy metals (Blaudez et al. 2000) can even open an ecological niche for the plant that it would not be able to grow in without being associated with ECM fungi.

Like in the formation of endomycorrhizae, ECM development involves a series of complex events. To elucidate such events, most research has employed simplified *in vitro* systems that are conducted to separate sequential phases (Horan et al. 1988; Martin and Tagu 1999; Fig. 2). In the first precontact phase, commonly called early phase, the fungus is attracted towards the root from a soil propagule by increasing hyphal branching and directional growth or from spores by inducing their germination. Fungal hyphae progressively colonize and adhere to the root epidermis (Fig. 2c, d). The docking process that consists of hyphae attachment to root epidermal cells starts with the formation of an "adhesion pad" through aggregation of hyphae (Jacobs et al. 1989). It has been proposed that the initial interaction of hyphae and roots during colonization occurs close to the root tip and that subsequently hyphae invade the root from root cap cells in- and upwards to the epidermis (Horan et al. 1988). Depending on the species, either a mantle of multiple layers of hyphae that multiply and differentiate (Horan et al. 1988) forms first around the root from which the Hartig net develops inward, or occasionally the Hartig net can establish first and the mantle forms afterwards (Nylund and Unestam 1982).

Once colonization of a certain root part is accomplished and the mantle and Hartig net are well developed, the nutrient exchanges take place between both partners in the Hartig net, leading to the functional mycorrhiza, called late phase (Fig. 2e). Furthermore, an equilibrium is established in terms of colonization: depending on plant and fungal species, the Hartig net attains a certain depth into the cell layers of the root (from the epidermis cells to cortex cells) and once colonization is accomplished the fungus does not penetrate any further. The equilibrium (biotrophic) phase, where efficient nutrient exchange occurs, may be maintained for a certain period of time, on the species involved (Smith and Read 2008), until the mycorrhiza ages and undergoes senescence (Dexheimer et al. 1986). It has been shown that ageing mycorrhizas may lose their fungal mantle, even if the Hartig net is maintained, and that their contribution to plant–fungus nutrient exchange diminishes (Al-Abras et al. 1988). The fungus can return back to a saprophytic lifestyle and invade senescent root cells (necrotrophic phase).
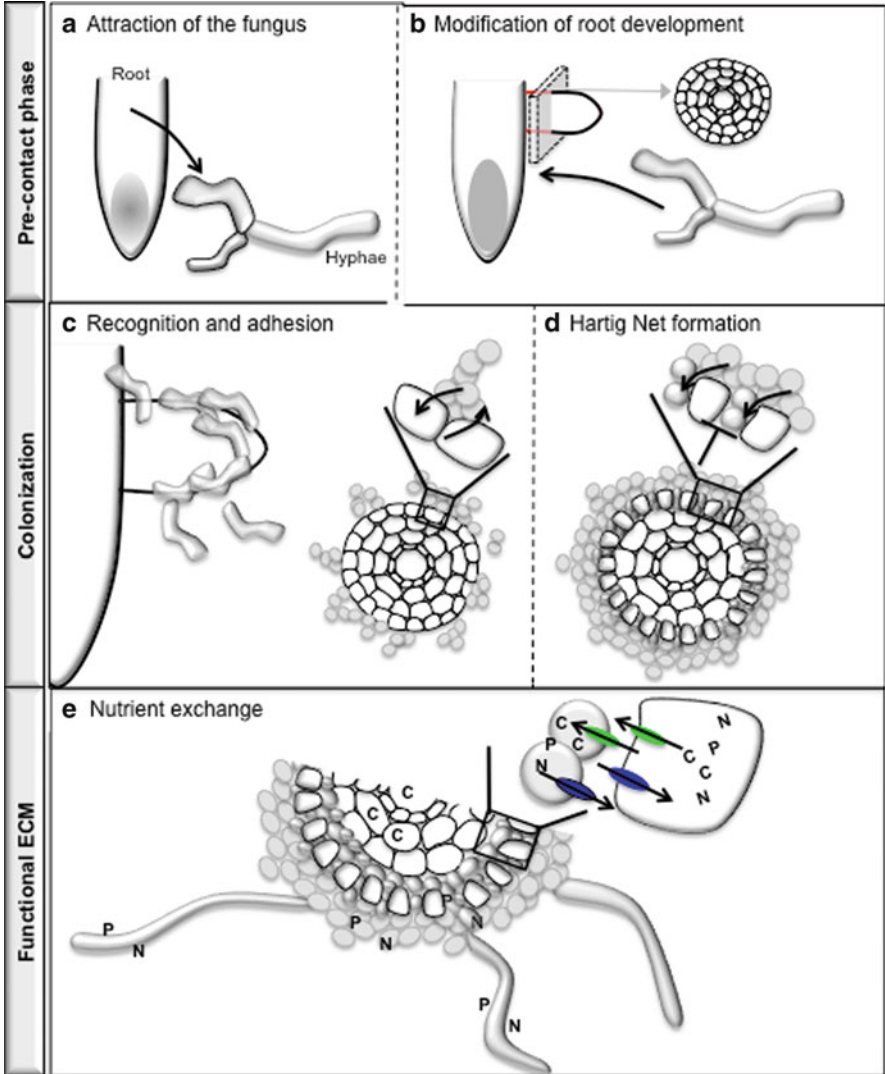
Fig. 2 Scheme of ECM development. ECM development can be separated into three main phases. (1) Signal exchange prior to physical contact, (2) colonization, and (3) nutrient exchange in the functional ECM. The precontact phase is characterized by a plant-to-fungus signalling to induce hyphae branching and attract the fungus (**a**) and to a fungus-to-plant signalling, which stimulates lateral root development of the plant (**b**). The colonization phase may be separated into a first recognition and adhesion of hyphae on the root (**c**) and of penetration of fungal hyphae in between epidermal (and cortex) cells of the plant (**d**), represented as a transverse root section. The functional ectomycorrhiza is characterized by active nutrient exchange (ammonium (N) and phosphate (P) from the fungus to the plant and sugars (C) from the plant to the fungus). Extramatrical hyphae explore nutrients in the soil and transport them to the mantle and Hartig Net where specific transporters insure the exchange

2 Reprogramming of Root Development

One of the observations when ECM were first described was the presence of numerous short and swollen lateral roots all over the root system (Fig. 1b). The presence of this type of root only during symbiosis illustrates the impact of the fungus on root system development.

The increase in lateral root formation is common for all studied ECM systems. Interestingly, these changes have been observed in response to endomycorrhizae fungi and also in response to bacteria (Olah et al. 2005), suggesting some common traits in the response of the plant host. Moreover, a stimulation of lateral root formation occurs during a change in nutrient availability, including N and P, suggesting a link between the mineral absorption by the fungus during the establishment of ECM and their effects on root architecture.

Recently, it has been observed in an in vitro system that the first visible modification in the induction of lateral roots, which emerge in response to fungal signals, from the parental roots starts in the very early phase of plant–fungus contact, even prior to physical contact between partners (Splivallo et al. 2009; Felten et al. 2009). These observations suggest the importance of a diffusible and/or a volatile signals in this change.

In the more advanced phases of ECM development, another striking change in root development can be observed, i.e., the formation of short roots. The detailed anatomical observations in *Eucalyptus globulus* in contact with Pisolithus clearly reveal a reduced root cap and a small apical meristem zone (Massicote et al. 1987). The differentiation of root tissues including endodermis, stele, and cortex starts closer to the apex. Our observations in poplar–*Laccaria bicolor* follow a broadly similar pattern to that of Eucalyptus (Fig. 1c). In conifer species, dichotomy of the root apical meristem can be observed, which results in short roots that branch just below their tip (Dexheimer and Pargney 1991; Laajanen et al. 2007).

More recently, it has been shown that root growth is guaranteed by the balance between stem cell proliferation and differentiation leading to a specific pattern of root organization. An auxin gradient, dictated mainly by auxin transport and the combinatorial action of some transcription factors, maintains the cell stem niche (Terpstra and Heidstra 2009). Interestingly, the auxin gradient is modified during the first stage of ECM formation (Felten et al. 2009). However, the potential function of transcription factors in the context of ECM is not established and fungal signalling molecules acting on the root apical meristem and columella initials are currently under investigation.

Another striking change in root development is the decay of root hairs in ECM (Ditengou et al. 2000). The absence of root hairs is thought to be due to the physical constraints of the fungal mantle around the root. Root hairs in plants are suggested to fulfill functions in nutrient (N, P) absorption (Bates and Lynch 2000). During symbiosis extramatrical hyphae efficiently take over these functions, so that root hairs are likely to become unimportant for proper ECM root nutrient provisioning.

However, it remains to be elucidated how the root developmental program is altered to halt development of root hairs.

3 Remodelling of Root Cell Walls

Cell walls are important features of plant cells that perform a number of essential functions, including providing shape to the many different cell types. Forming the interface between adjacent cells, plant cell walls often play important roles in intercellular communication. Because of their surface location, plant cell walls also play an important role in plant–microbe interactions.

In the context of ECM, hyphae aggregation and formation of the adhesion pad at the root surface involves recognition between both partners and synthesis of different cell surface structures. The formation of the new interface between the two partners plays an important role in the maintenance and the functioning of the ECM. Ultrastructural studies (Dexheimer and Pargney 1991) reveal that the interface is not formed by the superposition of the fungal wall and the cortical cell walls of the host plant. The parietal structure of the partners is modified to some degree, with, as a result, the production of a polysaccharide-rich cement involved in the cohesion of both partners and reinforcing the anchorage of the fungus to the plant cell surface (Fig. 1d). The presence of chitin has not been detected in this interface. This extensive modification of root cell walls is correlated with the induction of genes encoding cell wall synthesis, loosening and degrading enzymes that may facilitate the entrance of fungi between root cells (Duplessis et al. 2005). The same mycelium can colonize either the epidermal layer only or the cortical layer as well, depending on the host plant. This demonstrates that the host plant controls the fungal growth habit, but the molecular mechanisms are unknown.

Certain fungal cell wall polypeptides are specifically expressed in Pisolithus before or during early phases of Eucalyptus root colonization and are down-regulated later in the mature mycorrhiza (Duplessis et al. 2005; Hilbert et al. 1991; Hilbert and Martin 1988; Martin and Tagu 1995). These are termed "Symbiosis Related Acidic Polypeptides" (SRAPs). Another group of cell wall-related proteins that were overexpressed specifically at the same time points in *P*. *microcarpus* were hydrophobins. These molecules secreted by the fungus are considered to facilitate the intercellular penetration of hyphae (Nylund 1980) and consequently to have a considerable role in the construction of this new interface and in the communication between the two partners. Hydrophobins are small hydrophobic polypeptides with a conserved distribution of eight cystein residues. Only one class (class I) is found in Basidiomycetes (Wessels 1997). The immunolocalization of one of these hydrophobins, named HYDPt-1, from *Pisolithus tinctorius* (Tagu et al. 2001) reveals its presence in the surface of the hyphae in the mantle and in the Hartig net. The role of hydrophobins in ECM is not well known. It has been proposed that these proteins facilitate the communication between the two partners in participating in the construction of the surface.

In parallel, the establishment of ECM is accompanied by profound modifications in the host cortical cells walls. The orientation of cell growth is radial, suggesting a transformation of the parietal morphogenesis. However, no studies describe in detail the mechanisms involved.

These amazing processes occurring in the host root development implicate a complex signalling network between the two partners.

4 The ECM Fungus Perceives Plant Signals

It has been suggested that root exudates contain compounds that are recognized by fungi and that attract them (Horan and Chilvers 1990). In Eucalyptus, roots release the flavonol rutin that has been identified to stimulate growth of the fungus *P. tinctorius* at only a picomolar in concentration (Lagrange et al. 2001; Martin et al. 2001). It was furthermore shown that the cytokinin zeatin is able to induce branching in ECM hyphae (Martin et al. 2001; Gogala 1991). These factors might be the first communication between a plant and its symbiotic ECM fungus in the early phase (Fig. 2). Their attribution to the early phase can be made because it was shown that when a barrier is present between roots and fungi, compatible fungi will recognize the root and grow towards it (Horan and Chilvers 1990), suggesting that molecules present in root exudates are sufficient for this step. The fact that a flavonol is included in these "branching factors" is an interesting finding, as rhizobia bacteria are also attracted by flavonoids secreted by the plant (Aguilar et al. 1988). This indicates that similarities may occur between different symbioses concerning attraction of the microbial symbiont by the plant partner. Interestingly, the perception of plant exudates not only triggers development of hyphae but also accumulation of metabolites, such as hypaphorine, which may be involved in fungus to plant signalling and will be discussed below (Beguiristain and Lapeyrie 1997). Abietic acid extracted from Pinus roots was able to induce spore germination at a very low concentration (10^{-7} M) and this effect seems to be specific to the genus *Suillus* (Fries et al. 1987). Horan and Chilvers (1990) demonstrated that the presence of root-diffusible molecules is able to chemioattract ECM mycelia.

In the case of AM interactions, studies have revealed the role of strigolactone as a signalling compound to the hyphae aiding in branching and establishment of the mycorrhizae (Gomez-Roldan et al. 2008). Chemical signals released by symbiotic bacteria and by AM fungi are characterized; however, similar molecules have not been identified in ECM.

5 Hormone Signalling During ECM Development

Phytohormones (plant-growth substances) are metabolites that exist in extremely low quantities in plants, or also in microorganisms, and influence in a dose-dependent manner the development of all plant organs. In addition to the five

"classical phytohormones" auxin, ethylene, gibberellins, abscisic acid, and cytokinins (Kende and Zeevaart 1997), today, further molecules such as salicylic acid, brassinosteroids (Grove et al. 1979), and jasmonic acid (Creelman and Mullet 1997) have been assigned as phytohormones and further growth-modifying substances [e.g. strigolactones (Gomez-Roldan et al. 2008; Umehara et al. 2008)] are discussed as having phytohormone character.

In roots, different phytohormones are involved in lateral root development (Fukaki and Tasaka 2009), root meristem maintenance (Benkova and Hejatko 2009), root hair development, and elongation (Rahman et al. 2002; Pitts et al. 1998; Jones et al. 2009) and formation of the root cap (Wang et al. 2005). Auxin is a central element in root development, but, via crosstalk with auxin, other phytohormones also influence root development (Fukaki and Tasaka 2009; Benkova and Hejatko 2009). The fact that during ECM symbiosis root development is altered on all levels suggests that fungal signalling molecules can interact with or alter the root auxin levels that control these patterns. Because of their versatile nature and their production by plants and fungi, phytohormones are interesting candidates to mediate plant–fungus signalling during ECM development.

5.1 Volatile Phytohormones as Precontact Signals

Different studies have suggested that the first fungal signal perceived by the plant does not require any physical contact with the fungus (Splivallo et al. 2009; Felten et al. 2009, 2010). This conclusion has been based on studies that have demonstrated that LR formation in mycorrhizal or nonmycorrhizal plants is stimulated even when ECM truffle fungi or *L. bicolor* are spaced at a certain distance in a Petri dish, or are separated by a semipermeable barrier, from the plant. More strikingly, LR stimulation will even occur when only the exchange of volatiles and not soluble molecules between both partners is allowed (Felten et al. 2010). Therefore, the hypothesis was established that volatile molecules released by the fungus may be the first fungal signal(s) perceived by the plant. ECM fungi are able to produce volatiles such as the phytohormone ethylene (Rupp et al. 1989; Graham and Linderman 1980; Splivallo et al. 2009). Others have demonstrated that the fungus *Fusarium oxysporum* (a nonectomycorrhizal fungus) releases different jasmonates (Miersch et al. 1999). As diffusible molecules such as ethylene and jasmonates are known for their stimulatory effect on LR development (Regvar et al. 1997; Sun et al. 2009; Ivanchenko et al. 2008), they may be some of the key actors in fungal-induced LR stimulation during the precolonization stage. Splivallo et al. (2009) considered ethylene released by ECM truffle fungi as a stimulator of LR development in Arabidopsis as LR stimulation by these fungi was decreased in the ethylene insensitive Arabidopsis line *ein2*. However, in feeding experiments ethylene mimicked fungal LR induction only when applied together with exogenous auxin. Moreover certain ECM fungi, such as *P. tinctorius*, do not produce ethylene but nevertheless stimulate LR development (Rupp et al. 1989). This suggests that

ethylene may be part of the LR stimulating signals exchanged between fungus and plant during the early phase of interaction, but that it is not the only one. Interestingly, the exogenous application of methyljasmonate accelerated the first mycorrhizal contact of spruce roots and *L. laccata* (Regvar et al. 1997), which also suggests a possible role for methyljasmonate during the early phases of ECM establishment. It will be interesting to analyze whether jasmonates are produced by ECM fungi and to further study their effect on ECM development.

It is worth noting that experiments on ethylene levels during colonization have not distinguished between ethylene derived from the plant or from the fungus. The finding of Rupp et al. (1989) that there was an increase in ethylene levels released by roots colonized either with *L. bicolor* (produces ethylene) or with *P. tinctorius* (does not produce ethylene) suggests that the plant partner produces ethylene upon colonization. The same could be valid for jasmonates. Jasmonate and ethylene are usually associated with defense responses against necrotrophic pathogens or herbivore insects (Bari and Jones 2009; Plett 2010), but act as well in biotrophic (mutualistic) associations (Gutjahr and Paszkowski 2009). Consequently, they may be involved in the early defense response during root–ECM fungus contact.

5.2 The Role of Auxin During Early Contact Phase

Auxin, which is one of the major regulators of root development, has been suggested to be at the crosspoint of fungus–plant signalling and the modification of root development during ECM establishment (Felten et al. 2009; Splivallo et al. 2009; Laajanen et al. 2007; Raudaskoski and Salo 2008).

Slankis (1950) was one of the first to suggest a function for auxin released by ECM fungi in the development of ECM root tips. Since then, different research groups have addressed auxin production by ECM fungi in axenic cultures. Techniques ranging from thin-layer chromatography experiments (Ho 1987a, b) to quantification with ELISA techniques or IAA antibodies (Karabaghli-Degron et al. 1998; Rincon et al. 2003) and GC–MS analysis (Splivallo et al. 2009) have all found a very low production of fungal auxin in the nanomolar range (10–300 nM) for different fungi (Ho 1987a, b; Rincon et al. 2001, 2003; Reddy et al. 2006). While results from these studies indicate a large difference in the ability of individual fungal strains to produce IAA (Ek et al 1983; Rudawska and Kieliszewska-Rokicka 1997), even the highest amount of auxin released by certain ECM fungi appears too low to be responsible for early plant responses such as LR stimulation. This is confirmed by the results of Karabaghli-Degron et al. (1998), who demonstrated that LR induction in Norway spruce required 100–500 μM exogenously applied IAA and that below this concentration no effect was observed. However challenging roots with *L. bicolor*, which secretes only about 10 nM IAA, resulted in a visible LR stimulation. Splivallo et al. (2009) came to similar conclusions. They revealed an induction of the auxin response in roots during contact with truffle fungi, as visualized by *Arabidopsis thaliana pDR5: GFP*

lines. However, the fungi only released about 100 nM IAA into the medium, which when exogenously applied did not reproduce the effect of the fungus. Recently, we were able to show that when auxin and *L. bicolor* are simultaneously applied to *A. thaliana*, a cumulative effect is observed concerning LR stimulation, suggesting that fungal signals and auxin can work in synergy to induce LRs (Felten et al. 2010). Taken together these results highlight that fungal auxin in its very low quantity is unlikely to be the trigger of the early plant–fungus signalling. But what about internal root auxin?

We addressed this question in a study where we analyzed whether functional polar auxin transport inside poplar and *A. thaliana* roots was necessary for the fungus to stimulate LR development (Felten et al. 2009). Polar auxin transport occurs due to specific auxin efflux carriers (PIN proteins) situated at distinct sides of the plasma membrane of a cell that mediate the release of auxin from the cell. This directed transport permits auxin to accumulate at specific sites in the root, where it triggers, for example, lateral root primordium initiation or meristem maintenance (Fukaki and Tasaka 2009). Our results showed that polar auxin transport is required for the fungus to stimulate LR formation in poplar or Arabidopsis plants. Interestingly, one specific member of the PIN multigene family, PtPIN9 (orthologue of AtPIN2) was identified as necessary for fungus-induced LR stimulation. Interestingly, this member of the PIN family in Arabidopsis is responsive to jasmonates (Sun et al. 2009). PIN2 induction by the presence of the fungus during the precontact phase is therefore another step towards implication of volatile fungal molecules during the early fungus–plant signalling.

Moreover via their crosstalk with auxin, jasmonates and ethylene are also able to directly influence auxin biosynthesis in plant tissues (Fukaki and Tasaka 2009; Benkova and Hejatko 2009; Stepanova et al. 2007). The observed enhanced auxin response in roots during plant–fungus contact (Splivallo et al. 2009) may therefore be a secondary effect of directed auxin transport or auxin synthesis, not an accumulation of fungal auxin inside plant roots. In conclusion, fungal auxin is likely to play a minor role during the early signalling phase whereas other versatile signals, such as phytohormones ethylene and jasmonates, are likely to indirectly enhance auxin responses inside the root (Fig. 3).

5.3   Auxin as a Possible Signal During Cell Loosening and Hartig Net Development

Low amounts of auxin released by the fungus may be of major importance for root colonization once both partners come into close proximity and released auxin can accumulate inside plant tissues. As part of the "Acid-Growth Theory," auxin, as a weak acid, has been reported to have the ability to loosen the cell wall and to permit growth of cells (Rayle and Cleland 1992). Furthermore, Mensen et al. (1998) proposed that fungal auxin could reduce the peroxidase-catalyzed linkage of the
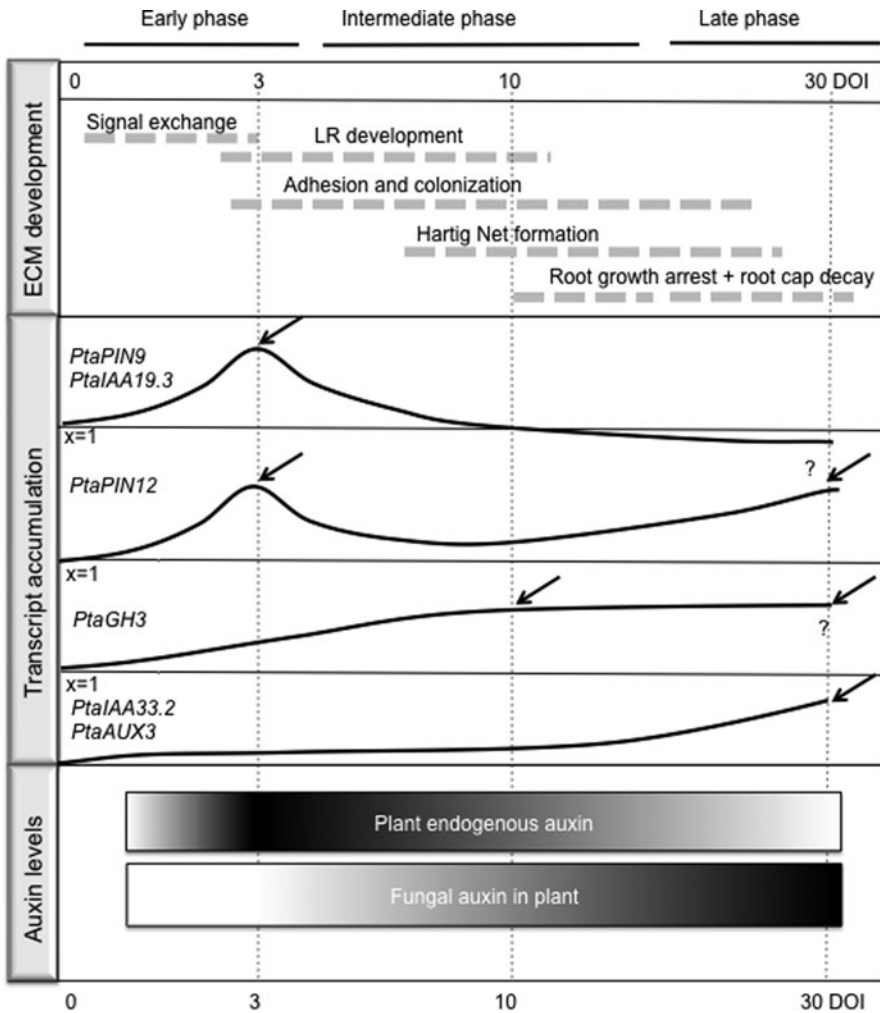
Fig. 3 Auxin homeostasis during ECM development

cell wall constituents, allowing hyphae to penetrate between the cortical cells to form the Hartig net. Results from Tranvan et al. (2000) illustrated nicely how IAA overproduction by the fungus *Hebeloma cylindrosporum* resulted in a quicker and deeper colonization of the root.

Reddy et al. (2006) as well as Charvet-Candela et al. (2002) had reported the differential expression of auxin-inducible genes *PpGH3-16* and *PpIAA88* in *Pinus pinaster* roots during colonization by *H. cylindrosporum* or *Rhizopogon roseolus*. Interestingly, their studies demonstrated no difference in transcript amount between roots challenged with wild type or the IAA-overproducing strain but a faster transcript change when the auxin overproducer was used. This indicates that the

accumulation of fungal auxin inside plant tissues triggers the rapidity and the final outcome of colonization.

In our own study (Felten et al. 2009; Fig. 3) we detected in poplar roots during colonization an accumulation of transcripts related to enzymes (IAA-amido-synthetases GH3) that are able to conjugate auxin to amino acids and thereby mediate dynamically auxin homeostasis (Staswick et al. 2005) and of auxin influx carriers, which import auxin into the cell. It may hence be possible that the plant actively removes fungal auxin from the apoplast and re-adjusts auxin levels. One reason for this could be to divert the free auxin into inactive conjugates or to degrade it. Indeed, Wallender et al. (1992) observed a decrease in auxin levels in mature ECM compared to noncolonized plants. This may be a result of the balance of auxin production by the fungus and auxin degradation by the plant in mature ECM where auxin levels may be drastically reduced to restrict further penetration deeper into plant tissues. Thus, auxin-homeostasis adjustment could be part of the mechanism that regulates the equilibrium of Hartig net colonization (Figs. 3 and 4).

Lastly, Karabaghli-Degron et al. (1998) and Rincon et al. (2001) addressed whether inhibition of polar auxin transport (acting on the plant and apparently not on auxin secretion from the fungus) impacted root colonization. They discovered that the polar auxin transport inhibitor TIBA completely inhibited *Eucalyptus* root colonization by *Pisolithus* and the inhibitor NPA caused an irregular colonization of roots. This finding points to the importance of polar auxin transport inside the plant when fungal auxin accumulates in plant tissues. This transport and redistribution of auxin is likely to be important in enabling colonization.

Also in cases of LRP emergence in the absence of any fungi, distinct auxin maxima that establish due to directed auxin transport have been shown to trigger cell wall remodelling enzymes and to permit cells to separate (Swarup et al. 2008). A similar mechanism may be involved to permit fungal auxin to activate cell separation during Hartig net establishment. Due to the lack of identification of auxin biosynthesis pathways in ECM fungi in contrast to other fungi (Reineke et al. 2008), hitherto no mutants deficient in auxin production are available. Those could confirm our hypothesis that fungal auxin is crucial for root colonization, but does not impact LR stimulation.

Interestingly, an antagonist of auxin has also been shown to be involved in ECM formation. This molecule is the tryptophane-derivative hypaphorine that is produced in large amounts by *P. tinctorius* upon ECM formation (Beguiristain and Lapeyrie 1997). It has been proposed that hypaphorine secreted by the fungus influences root endogenous auxin and thereby regulates symbiosis establishment (Ditengou et al. 2000; Ditengou and Lapeyrie 2000; Jambois et al. 2005). Hypaphorine had been shown to interfere with the actin and microtubular cytoskeleton (Ditengou et al. 2003) and with calcium fluxes (Dauphin et al. 2007), at least in root hairs. However, not all ECM fungi produce hypaphorine. Thus, a general mechanism based on this compound cannot be proposed. Nonetheless, it cannot be ruled out as fungi that do not produce hypaphorine may produce other indole compounds with similar activities.
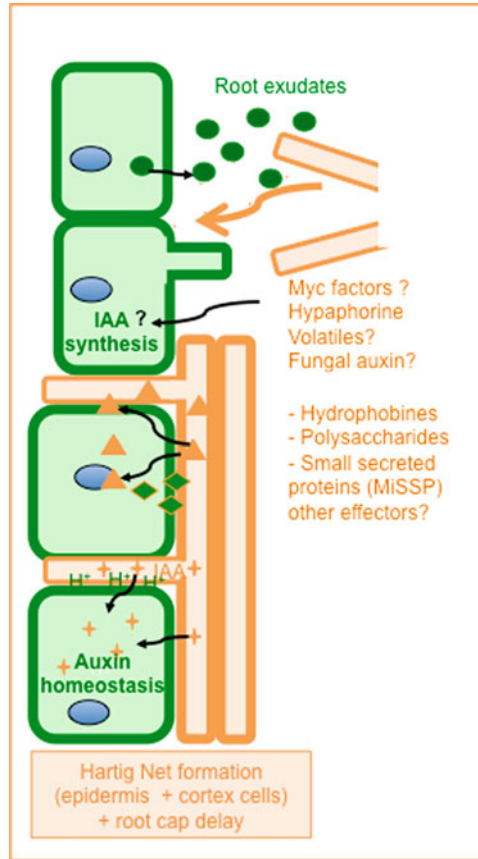
Fig. 4 Signal exchange during the developing ECM. The hypothetical events in the three phases of root colonization are related to root developmental changes observed during poplar root colonization with *L. bicolor*. During the early phase, root exudates induce hyphae branching and attract the fungus. The presence of the fungal signal molecules including volatiles stimulates auxin biosynthesis in plant epidermal cells. This process coincides with LR induction. During the intermediate phase, when colonization takes place, the fungus releases actively IAA through PGP-like carriers into the apoplast of root epidermis cell wall. Together with indole compounds released by the fungus and increased proton effluxes through plasma membrane H^+-ATPases. The induced acidification of the cell wall would facilitate cell spacing and penetration by the fungus. Cells start to decrease excess IAA through conjugation. In parallel, secreted fungal molecules including hydrophobines, polysaccharides and small proteins participate to the Hartig net construction. In the late phase, the fungus still releases IAA into the epidermis cell wall, but plants import this auxin actively and degrade it to prevent further apoplast acificiation and ongoing penetration of the fungus. Increased auxin degradation may decrease the total auxin content in the cells and also influence root growth arrest and root cap decay that were specifically observed in the late phase. Orange metabolites and proteins are of fungal origin and green ones of plant origin

Taken together, due to its low concentration, fungal auxin is unlikely to be the trigger of LR stimulation in the early phase of root–ECM fungus interaction. Sufficient fungal auxin accumulation in plant tissues is probably only possible

when the contact of both partners is close enough (mantle/Hartig net). In our model (Fig. 3), we propose a gradual change of plant and fungal auxin accumulation at the plant–fungus interface in respective phases of ECM development.

6 Secreted Fungal Molecules as Putative Effectors

Studies developed over the last years have demonstrated that secreted proteins also act as powerful effectors. The activity of these secreted proteins in the manipulation of host-cell structure and function has been broadly reported in many plant–microbial pathosystems (Kamoun 2007). Studies on signalling events in pathogenicity and symbiosis have highlighted a particular class of effectors corresponding to secreted small proteins (SSP). Transcript profiling analysis (Martin et al. 2008) has revealed that these SSPs, so-called Mycorrhiza induced Small Secreted Proteins (MiSSPs), are specially expressed in the Hartig net infection of the ectomycorhizal symbiont, *L. bicolor* in poplar.

Identification of the target molecules is crucial for a deeper understanding of the complex signalling pathways affected in host–symbiont interactions.

7 Evolution of Ectomycorrhizal Signalling

The beginning of ECM fungi evolution is dated to a large time period, probably about 180–130 mya, and it is possible that they arose at different occasions when saprotrophic fungi formed symbiotic partnerships (Hibbett et al. 2000; Alexander 2006; Moyersoen 2006). ECM fungi are therefore younger than endomycorrhizal fungi, which developed 450–350 mya, when plants first settled on land (Simon et al. 1993). The independent evolution of endo- and ectomycorrhizal symbiosis may account for the difference in their signalling mechanisms.

However, parallels can be drawn between ECM signalling and plant interactions with nonbeneficial microbes. Different transcriptome studies have revealed that a defense reaction arises in the plant upon early contact with the fungus, which is repressed at later stages of colonization (Duplessis et al. 2005; Le Quere et al. 2005; Sebastiana et al. 2009). Stress/defense responses involve an accumulation of measurable reactive oxygen species (ROS). ROS production during colonization of roots by ECM fungi is a known phenomenon and is likely to depend on the compatibility of the interacting partners (Gafur et al. 2004). Commonly during microbe–plant interactions an innate immunity response triggers the defense reaction in the plant partner. Innate immunity relies on the perception of a microbe/pathogen associated molecular pattern (MAMP/PAMP) by plant cells via receptors that activate the defense response (Boller and He 2009). But microbes and pathogens can release effectors (polysaccharide, proteins, phytotoxins, etc.) that repress this immune (defense) response. During ECM symbiosis an innate

immunity response has not yet been revealed, but as it is a rather general mechanism, we may suggest that it takes place upon root–ECM fungus interaction. The fact that during ECM symbiosis establishment the stress response is suppressed implies that the fungus secretes such an effector.

8 Conclusions

Even though several studies have addressed the specific signals that trigger ECM symbiosis establishment, the molecules and perception mechanisms involved remain largely unknown. Unlike bacterial or fungal endosymbiosis, where the recognition mechanism (ligand/receptor pair) between plant and fungus and parts of the downstream signalling cascade have been revealed (Stracke et al. 2002; Gherbi et al. 2008; Olah et al. 2005; D'Haeze and Holsters 2002), the early signalling in ECM symbiosis is still a black box. Thus, a clear scheme of factors involved in signalling, recognition, and colonization and their downstream signalling cascade cannot yet be drawn for ECM signalling. However, we propose some hypothetical events in the context of poplar/Laccaria bicolor interaction (Fig. 4). We make an attempt to assign them to specific phases in ECM development. Nonetheless, as data is fragmentary, it is possible that certain molecules are involved in more phases than the ones we have allocated them to in our models.

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