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The Roles of Auxins and Cytokinins in Mycorrhizal Symbioses

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Abstract

Most land plant species that have been examined exist naturally with a higher fungus living in and around their roots in a symbiotic partnership called a mycorrhiza. Several types of mycorrhizal symbiosis exist, defined by the host/partner combination and the morphology of the symbiotic structures. The arbuscular mycorrhiza (AM) is ancient and may have co-evolved with land plants. Emerging results from gene expression studies have suggested that subsets of AM genes were co-opted during the evolution of other biotrophic symbioses. Here we compare the roles of phytohormones in AM symbiosis and ectomycorrhizas (EC), a more recent symbiosis. To date, there is little evidence of physiologic overlap between the two symbioses with respect to phytohormone involvement. Research on AM has shown that cytokinin (CK) accumulation is specifically enhanced by symbiosis throughout the plant. We propose a pathway of events linking enhanced CK to development of the AM. Additional and proposed involvement of other phytohormones are also described. The role of auxin in EC symbiosis and recent research advances on the topic are reviewed. We have reflected the literature bias in reporting individual growth regulator effects. However, we consider that gradients and ratios of these molecules are more likely to be the causal agents of morphologic changes resulting from fungal associations. We expect that once the individual roles of these compounds are explained, the subtleties of their function will be more clearly addressed.

Key words: Phytohormones; Arbuscular mycorrhiza; Ectomycorrhiza; Cytokinin; *ENOD40*; Auxin; Root morphology

Mycorrhizal Symbiosis: Lessons from Ancestors

Mycorrhizal symbioses are mutualistic interactions between plant roots and fungi that in nature exist from germination of the seedling until the death of the plant. Plants exchange photosynthates (and often living space and the ability to sporulate) for water and mineral nutrients, particularly phosphate (in some cases also, the ability to survive).

Four broad types of symbioses are defined on the basis of the fungal class involved in the association and the morphology of the symbiotic structures. In this review, we will focus on two of the four types. These are the arbuscular mycorrhizal (AM) symbiosis (Figure 1), the most ancient symbiosis, which involves a few genera of zygomycete fungi and most land plants, and the ectomycorrhizal (EC) symbiosis (Figure 2), the predominant interaction for most temperate trees and some filamentous fungi (for example, truffles, boletes, *Amanita*). The other two major classes are the orchid mycorrhizas, involving some basidiomycetes and all members of the Orchidaceae, and the ericoid mycorrhizas, involving some

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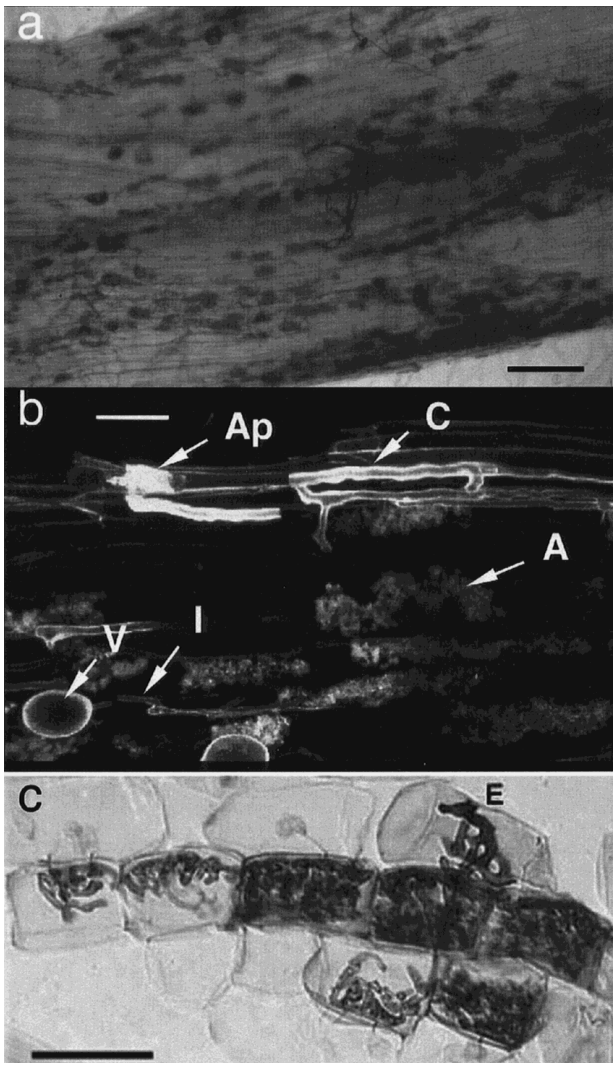

Figure 1. Morphology of the AM symbiosis. **(a)** Trypan blue–stained tomato root colonized with *Glomus mosseae* (Arum form). The small darkly staining areas in the root are arbuscules and vesicles, shown at higher magnification and clarity in panel **b.** The external hyphae are visualized as dark threads around the root. Bar = 250 µm. **(b)** Extended focus confocal microscope image of tomato root colonized by *Glomus intraradices. Ap,* appressorium; *C,* hyphal coil; *A,* arbuscule; *I,* intercellular hypha; *V,* vesicle. Bar = 50 µm. Image reprinted from Barker and others (1998a) with permission from Blackwell Science Ltd, UK. **(c)** Colony of an AM fungus spreading from the entry point (*E*) by convoluted hyphae in the inner cortex of an *Erythronium americanum* root (Paris form). This hyphal growth pattern is typical of roots without cortical air channels. Bar = 100 µm (photo courtesy of Dr. M. Brundrett).

ascomycete fungi, members of the Ericales and some Bryophyta (Smith and Read 1997). Additional variations on the mycorrhizal theme are also observed but have not yet achieved significant physiologic research status.

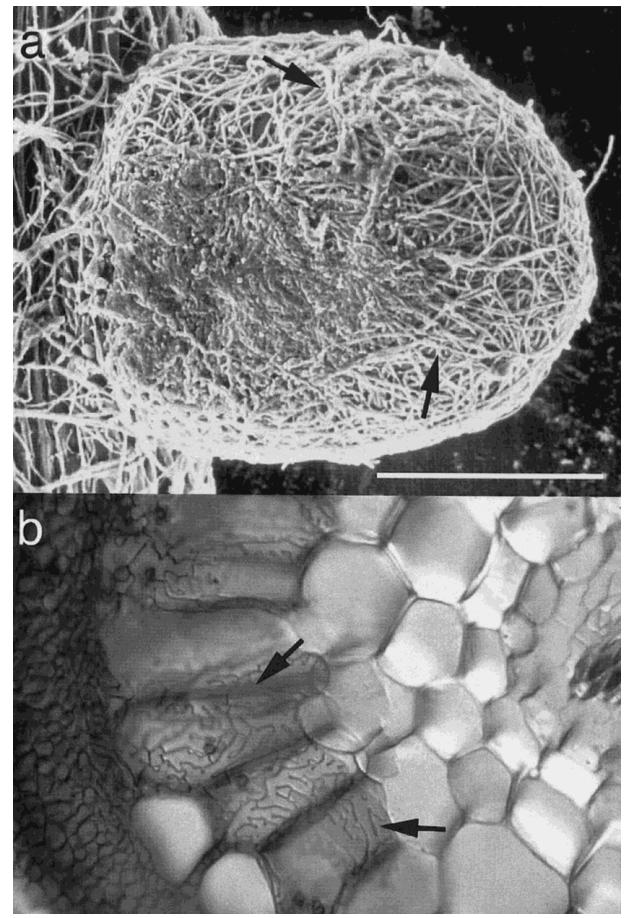

Figure 2. Morphology of the EC symbiosis. **(a)** Scanning electron micrograph image showing pine root colonization by *Pisolithus tinctorius.* Mantle hyphae have formed a dense covering on the short root surface (*arrows*) (Image reprinted from Piché and others 1983; photo courtesy of Dr.Y. Piché). **(b)** *Populus tremuloides* EC root cross section showing labrynthine Hartig net hyphae (*arrows*) around elongated epidermal cells (unknown fungus) (photo courtesy of Dr. M. Brundrett).

The mycorrhizal symbiosis is not a universal characteristic of modern plants (*Arabidopsis* for example, is resistant to mycorrhizal infection). However, although the proportion of fungal species involved is very small, an estimated 80% or more of plant families exhibit at least one type of mycorrhizal symbiosis (Smith and Read 1997). Evidence that the AM symbiosis co-evolved with the growth of plants on land, first proposed by Pirozynski and Malloch (1975), has been accumulating from fossil examinations (Remy and others 1994) and from molecular phylogenetic studies (Simon and others 1993). Although most hosts are able to complete their life cycles in the absence of the fungus, this interaction has been maintained throughout evolution until to-

day in most land plants, which is indicative of a highly successful partnership. Support is increasing for the idea that the AM symbiosis involves the expression of a set of genes, subsets of which have been commandeered in the evolution of more recent symbioses such as nodulation of legumes (Frühling and others 1997; La Rue and Weeden 1994; van Rhijn and others 1997). It is also possible that other biotrophic parasitic interactions such as root-colonizing nematodes can evade host defenses by mimicking AM signals (Tahiri-Alaoui and Antoniu 1996). Analysis of the AM symbiosis should highlight an ancestral gene set from the earliest stages of land plant evolution. Determining how members of this set have subsequently been recruited will provide an insight into evolutionary processes at the level of gene expression and may provide ideas for novel parasite resistance mechanisms (Barker and others 1998b). In contrast, ectomycorrhizal fungi appeared more recently (Selosse and Le Tacon 1998), and this symbiosis probably represents a modified saprotrophic or pathogenic interaction. Therefore, we have chosen to undertake a comparative inspection of the development of arbuscular mycorrhizas and ectomycorrhizas in the expectation that this will begin to indicate the extent of molecular flexibility underlying the establishment of compatible root-symbiont interactions.

Symbiotic Root System Signals: I Will Halt, You Go There

The general strategies of AM and EC root colonization are similar and involve spore germination, binding to the root surface, penetration of root apoplast, and branching and ingress of hyphae. Hosts and fungal symbionts exchange several rhizospheric signals (including phytohormones, see later), and this molecular communication is responsible for important morphologic changes in both hyphae and roots. For both AM and EC, only part of the root system is competent for fungal colonization. AM fungi preferentially colonize just behind the elongation zone of the root. In EC, the young root tips are most accessible to hyphal colonization (Smith and Read 1997). Thus, in both cases a physiologic or structural state of the root cell is necessary for sensitivity to fungal infection, and gradients of unknown morphogens (including phytohormones) may be involved in these processes, as proposed for nodulation by Hirsch and others (1997).

Germinating seedling roots are colonized by primary hyphae from germinating AM fungal spores or hyphae from neighboring colonized roots or root pieces. It is notable that root ingress by AM fungi occurs by means of appressorium formation (Figure 1b), as for many pathogenic fungi, which probably mimic this ancient symbiosis. In some pathogenic interactions, hormones such as ethylene regulate appressoria formation (Kolattukudy and others 1995). Appressoria allow the epidermal penetration of hyphae, which then grow into the cortex either by intercellular hyphae (Arum type, Figure 1a, b) or intracellular coils (Paris-type growth, Figure 1c). Highly branched arborescent structures called arbuscules (Figure 1b) grow into cortical cells (Arum) or may form on coils (Paris). Storage structures called vesicles are formed by some AM fungi (Figure 1b). All intracellular fungal structures are separated from the host cytoplasm by invaginated host plasma membrane. Metabolite exchanges between the two partners are generally assumed to occur at the arbuscular interface, but coils or intercellular hyphae may also participate in these processes (Smith and Smith 1996). External hyphae gather soil nutrients and form spores. Although there may be extensive ramification of fungal structures inside root cells (Figure 1a), changes to root system morphology are subtle and not extensively characterized. Reduced apical growth, reduced root shoot ratio, and increased formation of lower order lateral primordia have been reported (see later).

EC formation induces several evident morphologic changes, such as stimulation of rhizogenesis, the loss of root hairs, or the re-orientation of rhizodermal and cortical cells (reviewed in Smith and Read 1997). Rhizogenesis is followed, after root tip colonization, by a reduction of meristem activity, the consequence being the appearance of typical short mycorrhizal roots (Figure 2a). In some gymnosperms, dichotomous and coralloid structures may develop. These morphologic changes have long been attributed to the effects of phytohormones that are produced by EC fungi (see later). The filamentous EC hyphae bind to the root surface and form a compact sheath around it called the ectomycorrhizal mantle (Figure 2a). Some hyphae penetrate between root cells to develop an exchange zone called the Hartig net (Figure 2b), which could be considered the physiologic equivalent of the arbuscule. Fungal hyphae involved in the Hartig net are also highly branched (Martin and others 1999). However, intracellular penetration never occurs in ectomycorrhiza.

Progress in understanding the complexities of plant cell communication has been made with the use of molecular approaches, and the list of known plant growth regulators has essentially doubled in the past two decades (Franssen 1998). Explanation

of the integral role of both classic and modern phytohormones in mycorrhizal symbioses is in progress, and this review describes the areas of research and speculation in the recent literature. Although we are convinced that signaling molecules function by complex quantitative processes involving balances and gradients of components, much of the literature still describes phytohormones as having individual responsibility for particular responses. A fine balance between hormones and nutrient (phosphorus, carbon, or nitrogen) availability is probably important for controlled fungal cell differentiation in the mycorrhizal root. For EC, the roles of phytohormones versus carbon balance in the differentiation of ectomycorrhizal tissues were debated for some time, and a similar issue exists for AM, considering the roles of cytokinin (CK), carbohydrate, and P status in regulating the symbiosis (see later). However, it seems more and more evident that neither phytohormones nor nutrient status can act alone to mimic the morphogenic effects of mycorrhiza formation and that a combined effect of many parameters is necessary (Wallander 1992). Furthermore, one has to keep in mind that many other microorganisms—some of them producing phytohormones—cohabit in the rhizosphere and could play a helper role in plant-fungus interactions (Garbaye 1994). Here we will first raise the question of the putative role of phytohormones produced by the mycorrhizal fungi or the host plant on fungal biology. Then we will describe in more detail the data obtained on the roles of CK in AM development and the importance of auxins in EC differentiation.

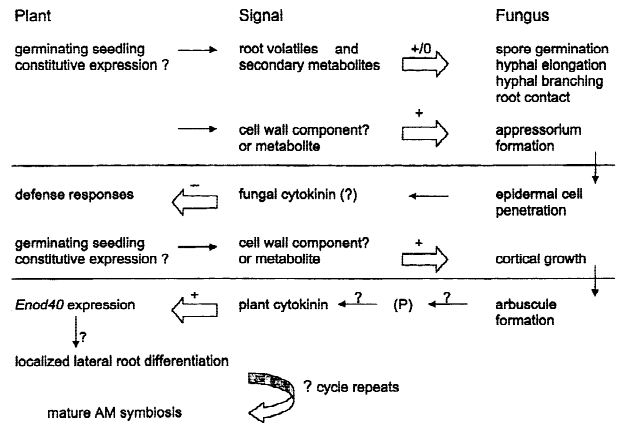

Figure 3. Signaling in the early stages of AM symbiosis. Putative pathway of events leading to AM fungal colonization of a germinating plant root system and the concomitant subtle changes to root morphology. Thin arrows indicate the flow of events. ? indicates a putative component or connection. Thick arrows with symbols indicate the effect of the signal. Horizontal lines separate stages in the pathway. In the first (*top*) stage, there is little evidence of AM fungal signaling to the plant. In the second (*middle*) stage, the partner's exchange signals to enable cortical growth, arbuscule formation, and localized first-order lateral root differentiation (*third stage*). In soil with low or patchy inoculum, repeating the cycle might enhance colonization potential by subtly altering root architecture, resulting in a fully colonized root system.

Phytohormones and Fungal Biology: The Dark Side

It is well recognized that root secondary metabolites, such as flavonoids or terpenoids (depending on the plant species), although not essential to AM symbiosis (Bécard and others 1995), certainly can affect spore germination, branching, and colonization (Figure 3). Carbon dioxide and/or other volatiles also assist in the spore germination processes (reviewed in Koide and Schreiner 1992; Morandi 1996). Isoflavonoids are structurally similar to estrogens, and estrogen-like binding sites that appear to have a role in regulating hyphal growth have been demonstrated in *Glomus intraradices* (Poulin and others 1997). Furthermore, a cDNA from *G. intraradices* (*Ginmyc1*) with sequence similarity to a steroid hormone receptor–binding protein has been cloned, and the gene products detected only in external hyphae, which is suggestive of a precolonization role (Delp and others 2000; G Delp, personal communication). Although the biologic function of *Ginmyc1* has not been determined, the possibility that it is part of a signal transduction cascade between roots and fungal hyphae is clearly worth investigating.

A second *G. intraradices* cDNA, *Ginmyc2,* has similarity to the *Arabidopsis* gene *SPINDLY,* which is involved in gibberellic acid (GA) signal transduction (Delp and others 2000). *Ginmyc2* is expressed both in external and internal hyphae (G. Delp, personal communication), but sequence similarities do not necessarily reflect functional similarity. Nevertheless, it is feasible that there is a role for GA in AM fungal biology if the report of GA-like compound synthesis by *Glomus mosseae* (along with auxin and CK-like compounds; Barea and Azcon-Aguilar 1982) is shown to be a general phenomenon. Recently, Blee and Anderson (1998) have put forward a model of arbuscule development, proposing that phytohormone (specifically GA) production by the arbuscular hypha as it enters the cortical cell initiates a series of events that enhance the carbon-sink activity of the infected cell. The events proposed include vacuolar invertase synthesis, acidification of the vacuole and the apoplast adjacent to the arbus-

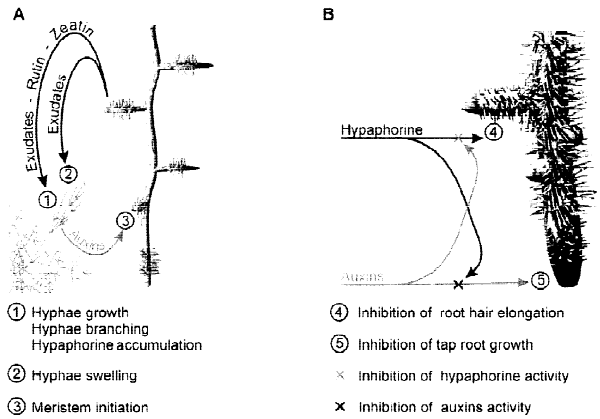

Figure 4. Summary of the major molecules exchanged and sensed between the two partners of the ectomycorrhizas and their effects on morphogenesis. **(A)** Effects of root exudates on hyphae morphogenesis during ectomycorrhiza formation. **(B)** *In vitro* effects of auxins and hypaphorine on root morphogenesis.

cule, and redirection of ER activities to the vacuole and plasma membrane. However, the literature has contradictory evidence on whether there is an increase in GA-like compounds in colonized roots (Allen and others 1982; Danneberg and others 1992). In addition, Slezack and others (2000) demonstrated inhibition of arbuscule formation by daily application of external GA. It should be possible to test Blee and Anderson's model by comparative research on arbuscule formation and function in GA-insensitive and GA-synthesis mutants in an amenable host plant such as tomato (Koornneef and others 1990).

Many EC fungi are able to produce *in vitro* most of the classic phytohormones (see Beyrle 1995). However, the question is whether phytohormones produced by mycorrhizal fungi function only in communicating with plant roots and in changing root morphology. There are some indications that plant CKs stimulate *in vitro* branching of ectomycorrhizal mycelia (Figure 4; see Gogala 1991; Lagrange and Lapeyrie personal communication). If confirmed, this would be evidence that fungal hyphae are receptive to at least some phytohormones. The lack of description of the role of phytohormones in fungal biology could simply reflect the fact that, *in vivo,* hormone actions are regulated by concentration gradients that are difficult to reproduce experimentally.

Phytohormones and Mycorrhiza Morphology: The Eclipsed Side

As explained earlier, root morphology changes when the roots are challenged with a symbiotic fungus. Because EC fungi produce phytohormones, a parallel has been proposed between the root modifications observed and phytohormone production. In the last 10 years, numerous data were obtained on CKs in AM and auxins in EC. Unfortunately, the effects of both hormones were not studied in parallel for each symbiosis, particularly because it is well known that CK/auxin balance is probably the main regulator (along with other hormones such as ethylene) of plant cell development. Thus, in the following sections, we will subdivide the data obtained for the two types of symbioses.

AM and Cytokinins: A Multifunctional Regulator?

In establishment of a mycorrhizal seedling, a subtle localized change in root morphology in response to the first symbiotic contact has been demonstrated. It consists of reduced apical growth, elongation of the colonized section, and increased (lower order) lateral root induction (Berta and others 1990; Torrisi and others 1999—but see Price and others 1989). The net result is increased root growth in the region of inoculum, which is likely to enhance colonization opportunities. Thus the subtle cycle of root morphology change repeats until a mature symbiotic root system has formed (Figure 3). That AM plants have enhanced CK accumulation in both shoots and roots, and that this is not a characteristic of pathogenic infections, has been firmly established (Allen and others 1980; Drüge and Schönbeck 1992; van Rhijn and others 1997). A pathway defining the roles of CK in establishment of the symbiotic phenotype can be proposed from the available data, but it is not yet unambiguous (Figure 3).

The recent collaborative research of the Hirsch and Kapulnik groups has involved comparative analysis of nodulation and AM symbioses and has considered the likelihood of overlapping gene expression sets and the role of phytohormones in both symbioses. In a landmark article, van Rhijn and others (1997) found that the early nodulin genes *MsENOD2* and *MsENOD40* were expressed in alfalfa roots in symbiosis with *Glomus intraradices,* but not in roots infected with the pathogen *Rhizoctonia solani.* The authors proposed that an increase in root CK was one of the signals directing the accumulation of the two *ENOD* gene products. The activity of CK in the induction of expression of two *MsENOD40* genes was confirmed by promoter analyses (Fang and Hirsch 1998; Hirsch and others 1997). We previously speculated that expression of *ENOD40,* in combination with increased CK content, is part of the mechanism

determining initiation of additional lateral root primordia in AM plants (Barker and others 1998b). The change in *ENOD40* gene expression may occur before the phosphorus nutrition of the plant is enhanced, but a very local influx of P from the young mycorrhiza cannot be ruled out. This is important to consider because it is not clear whether the CK is of fungal or plant origin, or is derived from both. Nonuniform application of P has been shown, in sterile soil culture, to increase the development of lateral roots (Drew 1975; Price and others 1989). There is also direct and indirect evidence that increased P nutrition results in enhanced CK production, presumably synthesized in the additional root primordia (Baas and Kuiper 1989; Danneberg and others 1992; Kuiper and others 1988; Salama and Wareing 1979). Further research is therefore required to provide an unambiguous pathway of cause and effect in AM root morphological change.

The other component of the interaction between AM fungi and roots that has been investigated quite thoroughly is the way that AM fungi evade or restrict the expression of defense responses in plant roots (for example, Gianinazzi-Pearson and others 1996). Altered expression of plant defense response genes in the symbiosis is apparent, but the mechanism regulating this is unclear. It has been proposed that there is a systemic suppression of defense responses in the mature symbiosis (Kapulnik and others 1996). Does the altered phytohormone balance of mycorrhizal roots explain these observations? In tobacco, Ginzberg and others (1998) found suppressed expression of two defense response gene family representatives (PR-1a and a basic chitinase) that was coincident with the increase in a ZR-like cytokinin in mycorrhizal roots compared with nonmycorrhizal controls. The authors speculate that the altered hormone balance is directly responsible for suppressed expression of some PR-protein genes, thus providing a mechanism for the observed lifetime compatibility of the fungus inside plant roots. Their results and speculations support those of Spanu and others (1989) for chitinase expression in mycorrhizal leek and of Lambais and Mehdy (1993) for β-1,3-endoglucanase and chalcone isomerase expression in mycorrhizal bean. These ideas, however, are inconsistent with observations that AM plants express enhanced resistance to root pathogens (Fitter and Garbaye 1994; Smith and Read 1997) and that transgenic plants constitutively expressing defense response genes are colonized normally by AM fungi (Vierheilig and others 1993, 1995). Research with mutant hosts unable to form a normal AM symbiosis indicates that the plant can activate these defenses right underneath AM appressoria (Gollotte and others 1993). *In situ* mRNA hybridization analysis of normal AM symbiosis shows that there is only a transient induction of members of defense response families as each arbuscule forms (Harrison and Dixon 1994). Considered as a whole, these data suggest that a systemic signal is not produced in plant roots once the root has become colonized with the fungi, but rather, that signaling between the fungus and the host is cell autonomous (Barker and others 1998b; Harrison 1999). It remains feasible that CK induced or produced locally by the AM fungal hypha is a component of this signaling mechanism.

The Mature AM Symbiosis: Certainly Not Just CK

Improved mineral nutrition in nonmycorrhizal plants tends to be associated with an increased root shoot ratio (Robinson 1994). Investigation of the plant growth response to mycorrhizal colonization has generally demonstrated that although overall plant growth attributed to improved mineral nutrition occurs, the root shoot ratios may actually decrease (Smith and Read 1997). Root growth may be reduced in the AM symbiosis because the fungus has become a significant C sink. This could have been tolerated in the evolution of the symbiosis because fungal hyphae have the role of fine roots (Smith and Read 1997). Note, however, that in modern agricultural settings, where nutrient additions are high compared with many natural ecosystems, the carbon cost can outweigh the benefits, and the symbiosis can become parasitic (Graham 2000). In addition to the effect on root growth of carbon reallocation, the increased flux of CK to the shoot (Allen and others 1980; Baas and Kuiper 1989) may enhance shoot growth, whereas root growth is unaffected by increased CK (Drüge and Schönbeck 1992; Van der Werf and Nagel 1996). The consequence of mycorrhizas on improved water relations of droughted plants is reflected in a reduction of ABA in roots and leaves (Duan and others 1996; Goicoechea and others 1997). Shaul and others (1999) suggested that enhanced viral susceptibility of AM plants might be due to increased CK in shoots causing suppression of defense response genes equivalent to that observed in AM roots. However, previous work on this topic suggested that improved P nutrition supported viral growth (Daft and Okusanya 1973). Finally, restriction of AM fungal growth in a mature symbiosis occurs by mechanisms affecting various steps in root colonization. Koide and Schreiner (1992) have suggested that this may be a consequence of altered

abundance of secondary metabolites mediated by changed P nutrition. Alternatively, McArthur and Knowles (1992) proposed that ethylene biosynthesis is repressed by increased phenolics in AM potato roots, thus enabling the symbiosis to develop, and that increased P in the mature symbiotic plant may counter this effect, enabling the plant to restrict further fungal colonization.

It is clear from this summary of the available experimental data that the precise mechanisms by which morphologic and physiologic changes are induced in AM plants have not yet been determined. The temporal and spatial cause and effect of CK abundance vs. P nutrition in the establishment of an AM symbiosis may be clarified using reporter gene constructs such as the transgenic alfalfa lines containing the *MsENOD40* promoter-*Gus* fusions (Hirsch and others 1997). In these plants, *Gus*-encoded enzyme activity after AM colonization would indicate increased cellular accumulation of CK, and concomitant isotope feeding studies might enable localized P accumulation to be determined. Development of similar transgenic plant tools for the other phytohormone classes will allow definition of the manner in which phytohormones are induced in the symbiosis. Furthermore, if a mutated or a natural variant of *Arabidopsis,* which is mycorrhizal, is ever found, it will be of major benefit to molecular AM research. Significant advances in understanding the roles of phytohormones in AMs can be expected in the next decade if careful application of molecular technology and examination of the older physiologic literature are combined.

Ectomycorrhizas and Auxins: Who Does What?

Auxins have been implicated in the formation of ectomycorrhizas for many decades, in part because of the morphologic changes that roots undergo during mycorrhiza formation. In the case of Pinaceae ECs, dichotomous branching of short roots occurs, resulting sometimes in the formation of coralloid structures made of the assemblage of numerous root branchings. Slankis (1973) reported that uninoculated pine roots treated with exogenous auxins developed similarly to EC roots. These observations led Slankis to propose the "hormone theory" of EC development, in which hormones were the unique drivers of this differentiation process. Furthermore, Slankis speculated that equilibrium existed between the level of nitrogen available for the mycelium and its production of auxins. He predicted that in the presence of a high level of nitrogen, production of tryptophan and, consequently, of auxins, was reduced. This theory has been criticized (for discussion, see Smith and Read 1997). For example, in comparing the concentration of IAA in EC and nonmycorrhizal pine roots, Wallander and others (1992) found lower levels of IAA in mycorrhizal than in nonmycorrhizal roots. In many other cases, treatment of roots with auxin alone did not mimic EC development. For example, Horan (1991) did not succeed in reproducing significant EC *Eucalyptus* root morphology by adding appropriate concentrations of exogenous auxin. However, modifications of root morphology by EC formation are slightly different between gymnosperms (specifically Pinaceae) and angiosperms. This may explain why the hormone theory, even if correct, cannot be generalized to other systems. More recently, Kaska and others (1999) demonstrated in a different pine species that dichotomous and coralloid branching of roots could spontaneously occur *in vitro.* Furthermore, auxin transport inhibitors (as well as ethylene) enhanced this morphogenesis. These data indicate that regulation of auxin concentration and its distribution in root meristems could be key factors for EC-like structures to develop. Inhibition of auxin transport causes a local increase in auxin concentration that then, probably through the action of ethylene, triggers EC-like root morphogenesis. The presence in the vicinity of tree roots of EC fungi producing auxins would probably modify the internal plant auxin balance in the same way to provoke root morphogenesis typical of ECs. However, the demonstration that EC fungi modify the concentration and/or distribution of auxins in the root remains to be done. The use of auxin transport inhibitors only suggests that auxins excreted by the fungus could be transported to the root cells (Karabaghli-Degron and others 1998; Rincon and others 2000).

The function of auxins in the biology of auxin-producing fungal mycelia is still under debate even if some data suggest that auxin may play a role in mycelium growth (reviewed by Tudzynski 1997). Mutants of the EC fungus *Hebeloma cylindrosporum* that overproduce tryptophan and auxins (Durand and others 1992) did not show significant changes in hyphal morphology or functioning. However, the ability of IAA-overproducer mutants to form ECs with *Pinus pinaster* was highly modified (Gay and others 1994). The mutants formed three to five times more mycorrhizas *in vitro* than the wild type. This phenotype was positively correlated with the quantity of IAA detected in the culture media of the different mycelia. Unfortunately, the lack of a sensitive method for auxin quantification impaired the capacity to draw conclusions on the level of auxins

reaching the root during colonization. Although the external morphology of the pine ECs obtained with the mutant and the wild-type mycelia were very similar, the internal organization of the ectomycorrhizal tissues was changed. Instead of a uniseriate Hartig net restricted to the outer half of the cortex within the wild type, a pluriseriate Hartig net, extending to the endodermis, was frequently observed with the IAA-overproducer mutant (Gay and others 1994; Gea and others 1994). Cytometric measurements of axial and radial diameters indicated that the shape of cortical cells was modified in roots infected by the mutant strain (Gay and others 1995). In some cases, mutant hyphae were able to penetrate inside living cortical cells. All these observations clearly indicated, for the first time, the involvement of IAA in EC morphogenesis. It is tempting to interpret the hypertrophy of the Hartig net as the result of the loosening of plant cell walls; auxins are known to affect cell wall structures by acidification of the apoplast (Salzer and Hager 1993). Analysis of cell wall surfaces did not show any differences in host cellulose and pectin or fungal chitin and β-1,3-glucan compound deposition between the different ECs, but more precise analyses are necessary to investigate the mechanisms of fungal ingress in the IAA-overproducing mutants. Furthermore, the fact that an IAA transport inhibitor such as TIBA inhibits the colonization of Norway spruce cortex by *Laccaria bicolor* seems to confirm the role of fungal IAA in Hartig net formation (Karabaghli-Degron and others 1998; Rincon and others 2000). In conclusion, the data obtained by Gay and others (1994, 1995) and Gea and others (1994) confirm in part the hormone theory proposed by Slankis (1973).

It seems evident that auxins cannot be the only molecule or signal involved in EC morphology. Auxins probably interact with other phytohormones (for example, cytokinins, ethylene) or with other plant metabolites in a concerted manner. The main regulator is probably not the synthesis of auxins but the finely tuned regulation of auxin concentration and distribution in the mycorrhizas. For example, Hampp and others (1996) showed that aspen trees that overexpressed bacterial IAA synthesis genes formed ECs *in vitro* with no differences compared with wild-type plants. Thus, by comparing the data obtained with an IAA-overproducing fungal (Gay and others 1994) or plant (Hampp and others 1996) partner, one can propose that the cells or tissues by which auxins are delivered to the root system play an important role in EC morphogenesis. The existence of gradients of concentrations of signal molecules is known to be essential for gene regulation. In the case of auxin signals in EC development, we believe strongly that coordinated control of auxin production and transport is the result of fine-tuning auxin activity in EC formation. The study of auxin-regulated genes during EC formation (Charvet and others 2000), analogous to the study of CK-regulated genes in AM symbiosis (Hirsch and others 1997), should help test this hypothesis.

IAA is a very unstable molecule and its pool in EC tissues is probably regulated by many conjugates able to deliver or sequester auxins rapidly when necessary. Thus, the presence of other auxinlike compounds has been investigated in ECs. By looking at indolic compounds regulated by mycorrhiza formation, Béguiristain and others (1995) described hypaphorine from exudates of *Pisolithus tinctorius.* The concentration of this molecule, which is a tryptophan betaine, is highly enhanced when hyphae colonize *Eucalyptus globulus* roots (Béguiristain and Lapeyrie 1997). Hypaphorine is able to regulate in eucalypt roots the expression of an auxin-regulated gene, *EgHypar,* which encodes a glutathione-*S*-transferase. This gene is also up-regulated during EC formation (Nehls and others 1998). Thus, the structure of hypaphorine (a tryptophan derivative) and its action on plant genes suggested that this molecule, which is abundant in *P. tinctorius* hyphae and exudates, might act as an auxin-like compound during EC development. However, additional experiments have demonstrated that this model is not correct (Ditengou and Lapeyrie 2000; Ditengou and others 2000). For example, hypaphorine has the capacity to reduce root hair elongation *in vitro,* which can be restored by application of exogenous IAA. Hypaphorine does not reduce taproot elongation, whereas auxin does, and, in the presence of both IAA and hypaphorine, taproot elongation halts (Figure 4). Hypaphorine, as well as *P. tinctorius,* can also counteract the effect of ethylene on apical hook formation of seedlings. This counteracting of ethylene effects is probably a consequence of hypaphorine interaction with IAA (Ditengou and others 2000). All these examples strongly suggest that hypaphorine antagonizes the activity of IAA on eucalypt roots and seedlings during EC fungal colonization. Because hypaphorine does not counteract the activity of nonindole synthetic auxins, it is suggested that hypaphorine interacts very early in the IAA perception and transduction pathway (Ditengou and Lapeyrie 2000).

It seems that important interactions between phytohormones and signaling molecules regulate auxin availability to the root cells, which triggers a pattern of root morphogenesis that is competent to form an EC. Future research will no doubt be directed toward the characterization of plants or

mycelia mutated in the production, sensitivity, or signaling of auxins and the analysis of the consequences on EC development.

Conclusion: Massive Attack Should Be the Key

As Harrison (1999) has commented, progress in understanding AM and EC, caused by the adoption of molecular tools (as anticipated by Smith and Gianinazzi-Pearson 1988) has been significant. In the next 5 to 10 years, progress in the comprehension of the roles of phytohormones in mycorrhizal associations will escalate because of the incredible development of functional genomics in plant and fungal species (for example, Voiblet and others 2000). The mass analysis of gene expression in mycorrhizal tissues will allow the identification of cellular processes affected by phytohormones. Furthermore, as some trees develop both AM and EC interactions, their study will allow a direct determination of any common subset of genes involved in both types of symbioses. Finally, the identification of plant mutants that are amenable to molecular-genetic characterization (Barker and others 1998a; Wegel and others 1998) should also reveal key genes involved in mycorrhizal symbioses. There is no doubt that among these regulatory genes, some, if not many, will be involved particularly in phytohormone production and in signaling. We look forward to this and other knowledge arising from continuing endeavors in scientific research.

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