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Morphological, biochemical and molecular changes during ectomycorrhiza development

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Summary. An ectomycorrhiza, a specialized root organ, is the result of a complex interaction leading to a finely-tuned symbiosis between a plant and a compatible ectomycorrhizal fungus. Ultrastructural observations combined with cytochemical and biochemical studies reveal that structural and metabolic changes in the symbiont cells lead to the final phenotype of the active ectomycorrhiza. In the present review these changes are interpreted as changes in gene expression and discussed within the context of ectomycorrhiza development. Recent genetic data indicate that the continued vegetative growth of the ectomycorrhizal hyphae and the root tissues, and their ability to switch to symbiotic organ formation, is basically controlled by developmentally critical genes. The activity of these 'symbiotic genes' during the differentiation of ectomycorrhizas is associated with extensive changes in the concentration of particular polypeptides and protein biosynthesis. The present state of knowledge about the developmental biology of ectomycorrhizas allows only speculation about the events during their development.

Key words. Symbiosis; ectomycorrhiza; ectomycorrhiza development; gene expression; ectomycorrhizins; protein patterns.

> *"Puisant mes forces aux sources des galaxies*
> *En buvant la sève des arbres"*
> M. Jonasz

An ectomycorrhiza, a specialized root organ, is the result of a complex interaction leading to a finely-tuned symbiosis between a plant and a compatible ectomycorrhizal fungus[25, 58]. The fungus supplies the basic enzymatic machinery for absorbing, translocating and assimilating major mineral ions (e.g., phosphate and inorganic nitrogen) plus a number of genes required for symbiosis. The plant provides the peculiar ecological niche that is necessary for fungal growth and development, including the completion of the sexual cycle, and it contributes the enzymes that assimilate the imported nutrients. The essence of the ectomycorrhizal symbiosis, as of most mutualistic associations, can be conceived as the formation of a physiologically integrated complex representing a distinct, a 'new', entity in itself, so that the fates of host and symbiont are markedly altered.

The differentiation of a complex structure, the ectomycorrhiza, from two relatively simple structures, the mycelium and the root tip, is an interesting model for developmental studies in higher fungi and plants alike. An understanding of how gene expression is regulated during the development of the symbiosis will be one of the major challenges of modern studies of the biology of the mycorrhiza. Like most developmental processes in eukaryotes, mycorrhiza development may be defined as a programmed sequence of phenotypic changes, resulting from differential gene expression, which is under temporal, spatial, and quantitative control. Mycorrhiza formation is the result of inherent genetic competence responding to specific environmental factors. These responses, in turn, promote changes at the cellular level, giving rise to new metabolic patterns.

We are not attempting a comprehensive survey in this review, and the references we will quote are offered for illustration, not as part of an extensive review. Rather, we will present some personal views and observations about the phenotypic changes which reflect the differential gene expression in the developing ectomycorrhiza. The ultimate goal of developmental studies is to define the formation of the symbiosis in genetic terms. However, developmental studies of ectomycorrhiza formation have mainly been descriptive, with development being described in morphological, anatomical, and biochemical terms. The following aspects will be dealt with; 1) signal exchange and changes at the cell surface; 2) changes of the cellular and subcellular organization during ectomycorrhiza differentiation; 3) changes in metabolic pathways in mycelia and ectomycorrhizas; and 4) changes in gene expression during mycorrhiza development.

Signal exchange and changes at the cell surface

Signal exchange in plant-microbe interactions, such as host/fungal pathogen or *Rhizobium*/legume symbiosis, or pathogenic associations involving Agrobacteria, involves the recognition and exchange of specific molecules (flavones, galacturonide fragments of plant cell wall, fungal β-glucans, fatty acids, and microbial pectin-degrading enzymes), produced by the host or the microbe or both, that trigger biochemical, physiological, and morphological responses that effect the development of the plant-microbe interaction[8, 24]. There is evidence suggesting an exchange of signals between the plant and the ectomycorrhizal fungus when the mycelium first arrives in the root zone. Unknown compounds including Melin's M-Factor[25], released by the plant roots, trigger events leading to the fungal infection of the host-root (see review by Gogala in this issue). In response to signaling by the plant, the fungus appears to synthesize factors that induce root branching and the disappearance of root hairs. Although these signals may not be specific for an ectomycorrhizal symbiosis, the early events induced by these signals permit the progression to later mycorrhizal ontogenic stages. Other plant signal substances are root lectins. These glycoproteins are apparently specifically involved in *Rhizobium*/legume symbiosis. In the final analysis, all these factors, and perhaps others, may play an interactive role in determining the specificity between host and infecting fungi.

Signaling molecules. In the saprophytic phase, propagules of mycorrhizal fungi apparently respond to the stimulation of root exudates. Germination of spores from four ectomycorrhiza-forming *Suillus* species (*S. granulatus, S. grevillei, S. luteus,* and *S. variegatus*) was induced by exposing the spores to exudates from roots of *Pinus sylvestris*. An active compound was isolated and identified as abietic acid, a diterpene resin acid[15]. Specificity in the activity of abietic acid is indicated by the fact that it does not induce spore germination in other mycorrhizal fungi. However, many ectomycorrhizal species exhibit a loose specificity since they respond to several types of germination activators from bacteria or other microorganisms[14]. Fungal cell fragments from *Rhizopogon* have been used to elicit phytoalexin synthesis in Douglas-fir tissues, which raises the question of the role of such molecules[3].

There has been much speculation that plant growth-regulating substances secreted by mycorrhizal fungi might play a prominent role in the symbiotic relationships between the mycosymbionts and their host-plants[25]. Ethylene, auxins and related derivatives induce anatomical and morphological changes (stunting and dichotomy) in roots of various trees. Ectomycorrhizal fungi similarly induce the formation of short, highly branched roots. Correlation of this change in morphology with hyperauxiny, and subsequently with secretion of the plant hormones, especially of IAA (indole-3-acetic acid), from the fungus has been demonstrated[16] (see also the review by Gogala in this issue). Similar alterations in root morphology may be induced by auxin treatment or by culture filtrates from the ectomycorrhizal fungus[16]. Unequivocal identification of IAA as a secondary metabolite from *Hebeloma hiemale*[56] and *Pisolithus tinctorius*[13] by spectrometric and immunological techniques has been reported. These fungi promote Douglas fir and pine growth when provided with tryptophan as an auxin-precursor[13]. The IAA-synthesizing activity of *H. cylindrosporum* varies among different isolates and among the progeny of one isolate of this species[17]. Differences in the IAA-secretion capacity between mutants of *H. cylindrosporum* are related to striking differences in the anatomical structures of the pine ectomycorrhiza in which they are involved. Isolates with a high level of IAA-secretion proliferate tremendously within the root leading to a hypertrophic Hartig net (Gay, personal communication). Dichotomous branching of pine roots in response to auxin was shown by Rupp and Mudge[57] to be due, at least in part, to auxin-stimulated ethylene production. Therefore, ethylene and auxin appear to be among the chemical signal-substances which control fungal growth within the root and some anatomical features of the mycorrhiza. Intraspecific variation in mycorrhizal structures of the *Laccaria bicolor/Pinus banksiana* association[64] may be related to differences in IAA-synthesizing activity among the various fungal isolates.

Modifications of the fungal wall. Early events in the interaction between plant root and ectomycorrhizal fungus are significant changes in fungal cell wall growth and differentiation and surface characteristics (see Gianinazzi-Pearson[18] for a detailed review). Production of glycoprotein-containing microfibrils by ericoid and ectomycorrhizal fungi in response to attachment to host-roots has been reported[6, 18, 38, 50]. The data suggest that interactions of mycorrhizal fungi with root cell receptors induce the formation of fibrils to anchor the fungus further to the plant cell surface. This is very similar to the role attributed to fibrillar sheaths of pathogenic fungi and to microfibrillae of symbiotic and pathogenic bacteria during the infection of host tissues[6, 8, 18]. These ultrastructural studies demonstrate a binding activity between mycorrhizal fungi and host-roots prior to the infection process. As in human parasitic fungi[48], the fungal cell wall may represent a reservoir of macromolecules which can modulate 'immunity' – compatibility – in the host. It has been suggested that the changing spectra of chemical components released by the pathogen or the symbiont during early infection may be involved with 'antigen' presentation to the host (Rhizobiaceae[24], lichens[49], rusts[47]). This, in turn, could generate different host responses. Appreciation of the importance of cell surface structure and chemistry in filamentous fungi and yeasts has led to new insights into the nature of the many interactions between these cells and their environment. A combination of biochemical and immunohistochemical techniques has revealed a highly complicated picture of the cell-surface changes in the rust fungi/plant associations[47]: certain carbohydrate structures appear to be specific to given stages of hyphae differentiation. In striking contrast to this impressive volume of literature, there is a paucity of information on the structure and composition of the envelope of ectomycorrhizal fungi[6, 18, 25]. Two lectins of different specificity (one recognizing lactose and another recognizing fucose) were purified from the mushroom *Laccaria amethystina* by affinity chromatography[23]. A search for lectin-dependent interactions between roots of pine and spruce and the neutral polysaccharides of the cell wall of the ectomycorrhizal *Suillus variegatus* and *Piloderma croceum* was unsuccessful[52]. However, the lack of interaction may have been due to the fungus used in these studies having been grown in vitro. Plant-secreted factors may be necessary to induce the synthesis of cell surface lectins or glycoproteins. This hypothesis is supported by the higher lectin binding in the ectomycorrhizal roots of the *Alnus crispa/Alpova diplophloeus* association, as compared to non-mycorrhizal control roots[44]. In the Hartig net region of mycorrhizal roots, the fucose-, mannose-, and N-acetylglucosamine-specific lectins bind intensely to the host cell wall, especially the wall ingrowths, and to adjacent fungal walls. In contrast, a weak lectin labeling was observed in the cell wall of non-mycorrhizal roots. The carbohydrate residues appear to be parts of glycoproteins.

It is evident from these various studies that the contact of the mycorrhizal hyphae with the host epidermal cells plays a key role in initiating and regulating the interaction between the symbionts. Attachment of cells and molecular interactions within the cell walls may act as keys to unlock further stages of mycorrhiza differentiation. This raises several intriguing questions: 1) What are the biochemical signals generated at the cell membrane or cell wall to initiate mycorrhiza formation? 2) How do these signals reach the intracellular machinery? 3) How do the signals generated by various stimuli 'cross-talk', in order to produce a well-coordinated message? This is an area of high-priority research that promises important results.

Changes in the cellular and subcellular organization during ectomycorrhiza differentiation

Hyphal tip growth and mycorrhiza initiation. Just as the key to hyphal growth lies at the tip[20], the development of the mantle and Hartig net is linked to pivotal events at the hyphal tip. Mycelium located on the surface of the primary root was uniform in diameter and had frequent clamp connections, while that forming the early stages of the mantle on first-order mycorrhizal laterals was often greatly enlarged and multi-branched[46]. Kottke and Oberwinkler's observations[30] support the view that morphogenetic changes take place upon contact of the hyphae with living cortical cells, which is pivotal for initiation of mantle formation and Hartig net construction. It is not known whether the topology of the root surface influences the direction of the hyphae, as was shown in rust fungi[47]. Progression from the strongly rhizomorphic outgrowth of the free-living mycelium to the plectenchymatous structure of the ectomycorrhizal sheath and the coenocytic Hartig net hyphae is associated with a lack of septation, a loss of apical coherence, and intimate juxtaposition of hyphae. As pointed out by Gregory[21], differentiation of vegetative organs involves a change of mode in the mycelium, which has to fulfil a different function and comply with different laws. A wide variety of exogenous and endogenous factors affect morphogenesis of vegetative organs in higher fungi[54]. Exogenous factors cannot themselves explain the intrinsic capacity for differentiation. It seems more adequate to view their effects in terms of interactions with endogenous control mechanisms. There are arguments in favor of taking the view that high nutrient levels, such as those occurring in the apoplast of root cortical cells, cause differentiation of mycelium[30]. In addition to major nutrients, several metabolites (IAA, fatty acids, amino acids) have been implicated in the initiation of plectenchymatous structures in agarics[54].

Changes in the cell ultrastructure. Ultrastructural analysis of ectomycorrhizal ontogeny conducted on eucalypt[45], alder[46], and spruce ectomycorrhizas[30, 31] have indicated that the cells involved in the interface between the symbionts undergo extensive changes during ontogeny. The hyphae penetrate between epidermal and sometimes cortical root cells, inducing changes in the cell wall architecture, which presumably involve pectin hydrolysis in the middle lamella[40]. Changes in the host cell wall may involve what appear from ultrastructural observations to be degradative events, or the synthesis of new cell wall polymers to produce wall ingrowths. In addition, regular, highly organized wall branchings may be induced in the Hartig net hyphae[31, 32, 44, 45, 50]. Thin sections of the interface have revealed that numerous mitochondria, lipid bodies, dictyosomes with proliferating cisternae, and extensive endoplasmic reticulum are contained within the coenocytic hyphae of the Hartig net. All these features are indications of a highly active anabolic state, and are involved in the net biosynthesis of new materials (i.e., biopolymers). In this region of the Hartig net, the hyphae contain several nuclei that may be in the process of dividing. Abundant invaginations of the fungal plasmalemma have been observed[44]. These membrane structures allow ions and metabolites to pass at a high rate between adjacent cells[32], providing the anatomical basis for intercellular communication and the local coordination between the symbionts. Endoplasmic reticulum with numerous ribosomes is indicative of a high level of biosynthesis of secreted proteins. These secreted proteins may include acid phosphatases and enzymes involved in cell wall degradation (i.e., pectate lyase, peroxidase and polygalacturonate lyase). The clustering of organelles within the Hartig net, presumably through modulation in the organization of the cytoskeleton[53], may facilitate the more efficient transport of substrates among these organelles and between them and the symbiotic interface.

The final structure of ectomycorrhizas is a product of complex interactions between host and fungal genomes that also involves environmental factors. Information on the ultrastructural basis of ectomycorrhizal ontogeny enables researchers to ask pertinent questions concerning the biochemical and morphological differentiation during the establishment of an ectomycorrhizal association. For example, many of the observed cytological modifications require synthesis of different kinds of polypeptides (e.g., membrane proteins, ribosomal polypeptides, and structural glycoproteins). Therefore, ultrastructural studies may help to identify the nature and the function of the symbiosis-specific polypeptides (see below).

Changes in metabolic pathways

Little work has been done on levels of enzymes and metabolic fluxes in ectomycorrhizas at early stages, possibly because of the difficulty of obtaining sufficient tissue for analysis. However, the importance of this early period of mycorrhiza development should be emphasized, since during this period the tissues are changing from a metabolite sink to a nutrient source for plant growth. Studies of metabolite levels and enzymes in several types of ectomycorrhizas have revealed differences between different stages of mycorrhiza development and also between different symbiotic tissues (e.g., extramatrical hyphae, Hartig net mycelium). These studies, summarized below, raise the hypothesis that the conversion of free-living partners to symbionts induces different enzymes and metabolic pathways.

Carbohydrate metabolism in ectomycorrhiza. The pathways of hexose catabolism in ectomycorrhiza and in free-living mycelium have been investigated in detail. Evidence supporting the catabolism of glucose by the Embden-Meyerhof-Parnas pathway and the pentose phosphate shunt has been reported in *Hebeloma* sp. spruce mycorrhiza[2, 5]. The contribution of the pentose phosphate pathway to respiration is higher in the fungal component than in the plant tissues. The fact that the pentose phosphate pathway activity was even higher in root tissues colonized by the fungal cells (mantle and Hartig net) than in extramatrical hyphae suggests that the contribution of this oxidative pathway is stimulated when the symbiotic fungus is associated with a root. Whether there is an increase in the activity of the pentose phosphate pathway enzymes or changes in the respective polypeptide amounts during the ectomycorrhiza formation must await further immunochemical analysis. The tricarboxylic acid cycle has been reported to occur in free-living ectomycorrhizal fungi and beech ectomycorrhizas, but so far no comparative studies have been reported[25, 43]. It has been suggested that the presence of the fungus reduces the rate of the basal respiration (minimum maintenance respiration) of plant tissues on which it grows[41]. It might achieve this decrease in the respiration rate by reducing the enzymatic work that the root tissues must carry out. The mycelium absorbs, assimilates, and transports a large part of nutrient input. As a consequence, the maintenance of the redundant enzymes in the plant tissues would seem to be a waste of nitrogen and energy. This hypothesis is strongly supported by our results on polypeptide biosynthesis[27,28] (see below in *Changes in the patterns of polypeptide synthesis during ectomycorrhiza development*). Regardless of the biochemical processes involved, it is clear that ectomycorrhizal colonization can reduce maintenance costs.

Nitrogen assimilation enzymes in ectomycorrhiza. Developmental regulation of the pathways for N assimilation in ectomycorrhizas is beginning to be elucidated. There have been tracer experiments and enzymological studies in various types of ectomycorrhiza, aimed at obtaining information about the regulation of the pathways involved[43]. In *Fagus-Lactarius* ectomycorrhizas, assimilation of ammonium involves the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle, rather than the fungal NADP-linked glutamate dehydrogenase (NADP-GDH)[10,42,43]. In beech ectomycorrhizas, the low activity of the fungal NADP-GDH is a consequence of the low GDH polypeptide content[10]. In contrast, the NADP-GDH pathway is the main assimilatory pathway in free-living ectomycorrhizal fungi[43]. Similarly, fungal aspartate aminotransferase activity is no longer detected in ectomycorrhizas of *Picea excelsa – Hebeloma* sp., *Pseudotsuga douglasii – Laccaria laccata, Fagus sylvatica – Paxillus involutus*[7]. Vézina et al.[62] further reported that the levels of activity of N-assimilating enzymes are modified in jack pine roots upon mycorrhiza formation. Ectomycorrhiza differentiation, therefore, alters the biosynthesis of N-assimilation enzymes, but the nature of these changes depends on the plant and fungal associate. The distribution of the N-assimilating enzymes between fungus and root has many implications for the transport mechanisms at the symbiotic interface[59]. The mechanisms underlying the regulation of the ammonia assimilatory enzymes in ectomycorrhizas are not yet understood. The fungus possibly metabolizes the host amino acid pools, and ultimately the fungal NADP-GDH gene is repressed by glutamine or other amino acids, either supplied by the plant or from accumulations in the storage layers of the fungal sheath. The assimilation of N in filamentous fungi is a highly regulated process[12]. Certain N sources, such as glutamine and glutamate, are preferentially utilized by these fungi, and synthesis of the enzymes needed to utilize nitrate and ammonia occurs only in the absence of the favored nitrogen sources, i.e., such synthesis requires the lifting of nitrogen catabolite repression[12,26]. Glutamine is the key metabolic effector for nitrogen repression in *Neurospora crassa* and *Aspergillus niger*, and was also shown to repress the synthesis of the NADP-GDH in *Cenococcum geophilum* (Martin, unpublished observations). So far, differential rates of turnover of the GDH polypeptide or mRNA in beech ectomycorrhizas have not been distinguished from differential transcription of the GDH gene. Because the regulation of N-assimilation pathways appear to differ between mycorrhizal roots of spruce and beech, further work is in progress to ascertain the role of host species in the expression of the fungal GDH pathway in ectomycorrhizas.

As a consequence of alterations in N-metabolism enzymes, the profiles of free amino acids in roots are also modified by the differentiation of the ectomycorrhiza. Krupa et al.[35,36] reported a significant increase in amino acid biosynthesis in various ectomycorrhizas. Decrease in arginine content with a parallel increase in glutamate and glutamine was the most conspicuous result. Recently, Vézina et al.[62] also reported significant changes in free amino acid proportions during ectomycorrhiza formation.

Phosphate metabolism in ectomycorrhiza. Host control over fungal development and phosphate metabolism has been well documented in endomycorrhizal, ericoid and ectomycorrhizal associations[25,58]. The presence on mycorrhizal fungi of nonspecific surface acid phosphatases capable of hydrolyzing a number of organic P compounds has invited speculation[18,25,60]. Mycorrhizas may contribute to the P nutrition of the host plant through the enzyme-catalyzed hydrolysis of organic sources otherwise unavailable to the higher plant. Pi starvation also induces a wide array of metabolic effects that modify plant growth at the organ, tissue, and cellular levels. Goldstein et al.[19] have recently presented evidence for the occurrence of a phosphate-starvation inducible rescue system in higher plants. One functional aspect of such a system involves the secretion of acid phosphatase in the rhizosphere. Some of the metabolic changes resulting from Pi starvation result from changes in gene expression, and the term Pi stimulon has been coined to refer to the entire set of genes responding to Pi starvation. The regulation of mycorrhizal acid phosphatases by inorganic phosphate has been investigated in several associations[18,25,58].

Most of these studies confirmed that increased phosphatase activity is associated with P deficiency. Both inducible and repressible phosphatases occur in ectomycorrhizal fungi. Cytochemical studies of eucalypt ectomycorrhizas have shown that acid phosphatase activity was localized at the surface of the fungal plasma membrane four days after inoculation[38]. Plasma membrane activity was detected when the hyphae were close to the roots, whereas the enzyme activity was barely detectable in hyphae distant from the roots. Straker et al.[60] have observed the disappearance of the phosphatase activity localized on the surface of external hyphae once hyphae have penetrated the *Erica hispidula* root cells. Using immunochemical techniques, it will be possible to define whether the differences in the levels of the hyphal phosphatase activity during the colonization are only the result of a modulation of enzyme activity or whether the host plant directly regulates the enzyme synthesis[60]. In fungi and yeasts the phosphatase system is complicated, being composed of numerous structural and regulatory genes[65]. Expression of the genes for repressible acid phosphatase (rAPase) and the low K_m-Pi transport system are repressed by Pi in the medium. Signals for the presence or absence of a repressive amount of Pi are conveyed through a system composed of at least five gene products. Because the rAPase presumably plays a central role in mycorrhiza biology, cloning its gene has a high priority. Because fungal rAPases share a high degree of nucleotide sequence homology in several regions of the gene, a reasonable strategy is to exploit these to identify genomic and cDNA-clones for rAPase from ectomycorrhizal fungi using heterologous DNA probes from yeasts or *Neurospora crassa.*

Intermediate metabolism. Phenolic compounds are synthesized in the epidermal cells of eucalypts in response to mycorrhizal colonization[29]. A few hours after inoculation, phenolic deposits appear within cap cells of *Eucalyptus globulus*, even before attachment of *Pisolithus tinctorius*. Thus, phenol synthesis is one of the first reactions of the root cells during mycorrhiza establishment. Similar deposits are also induced by *P. tinctorius* in *E. pilularis*[45] and are a consistent feature in pine ectomycorrhizas[40].

In summary, the development of ectomycorrhizas rapidly induces changes of nutrient fluxes and enzyme activities in many metabolic pathways. Many of the metabolic switches observed in mycorrhizas require changes in gene expression. However, the changes from anabolism to catabolism may also depend upon the pre-existence of catabolic enzymes, compartmentalization of anabolic enzymes, and the action of organelle membranes. A better and broader picture of the metabolism and biochemistry of developing ectomycorrhizas is required, and comparative details are essential. This implies the need not only for a comparison between different species (fungus and host-species) in order to identify causal events, but also for comparisons between tissues of the mycorrhiza (mantle vs. intercellular mycelium, cortical parenchyma cells vs. stele cells).

Changes in gene expression during mycorrhiza development

One of the central problems of developmental biology is to determine the nature of mechanisms that control the activation and expression of developmentally critical genes that are indispensable for the orderly progression of development. The brief account of morphological, physiological, and biochemical modifications experienced by symbiotic partners of ectomycorrhizas serves to indicate that the continued vegetative growth of the ectomycorrhizal hyphae and root tissues, and their ability to switch to symbiotic organ formation, is basically controlled by such developmentally critical genes or 'symbiotic genes'. The notion that the symbiotic genes act as master switches that control hyphal and root development is strengthened by the observed effects of certain mutations in these genes leading to non-mycorrhizal strains of fungi.

Developmental genetics. One of the major contributions of genetics to an understanding of mycorrhiza development lies in the provision of well-defined, morphological mutants. The isolation and characterization of mutants, which on the whole have some profound effect on phenotype and thus are easily detected, are starting to make an important contribution to our understanding of developmental processes in mycorrhiza[11, 34, 64]. Natural and experimental mutations identify genes that play important roles in the processes of interest and can help to dissect the complex mechanism of mycorrhizal ontogeny into smaller parts. Mutations can also identify specific gene products and determine their functions.

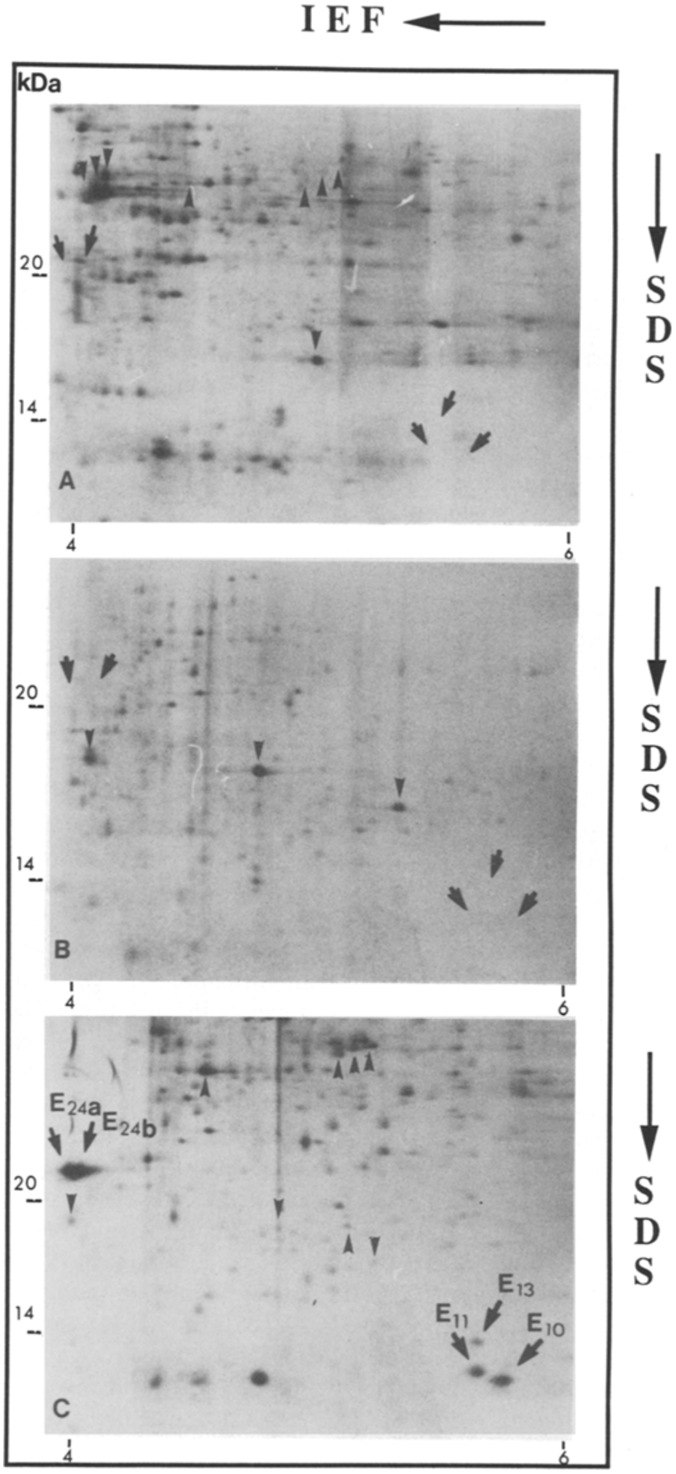
Spores of ectomycorrhizal fungi are monokaryotic and give rise to monokaryotic hyphae, but the hyphae of the fungal mantle are composed of binucleate mycelium. Detailed studies on *Tuber melanosporum* and *Suillus granulatus* indicated that ectomycorrhizas were only formed by heterokaryons which developed after fusion of compatible monokaryotic hyphae[22]. Similarly, Lamhamedi et al.[37] revealed that for *P. tinctorius*, the heterokaryotic state was necessary for the full expression of its ectomycorrhizal ability. The two interacting monokaryons must carry different alleles of their incompatibility genes in order to form a dikaryon. As a result of this genetic difference, not only are there differences in mycelial morphology and physiology[17, 33], but there is also a conspicuous difference in the ability of the mycelia to produce mycorrhizas under specific environmental conditions[34, 37]. These data suggest that the loci of the incompatibility genes may control the expression of morphogenetic sequences related to the formation of ectomycorrhiza. On the other hand, recent genetic studies using sib-selected homokaryotic hyphae from *Hebeloma cylindrosporum*[9] and *Laccaria bicolor*[34] demonstrated that monokaryotic mycelium was able to form mycorrhizas under laboratory conditions. In the case of *L. bicolor*, an extensive range of variation in ectomycorrhizal ability was observed[34]. Some of the monokaryons were strongly ectomycorrhizal, others formed no ectomycorrhizas, and some quickly lost their ability to colonize host roots. Processes regulating ectomycorrhiza formation are obviously under the control of genetic determinants. Kropp et al.[34] concluded that "the inheritance of mycorrhizal ability will probably turn out to be complex, with different characters controlled by major genes, polygenes, or cytoplasmic factors". In addition to this inherent genetic competence, many factors such as the age of the fungal culture and the phenological and the physiological state of the root certainly affect the expression of the ectomycorrhizal ability. Fungal mutants can be blocked prior to initiation of mycorrhiza, as exemplified by *L. bicolor* CRBF 0348[34, 64]. Wong et al.[64] later made a detailed anatomical study of ectomycorrhiza formation using morphogenetic variants which are blocked in the process of mycorrhiza development. Such mutants undergo morphological changes that signal the onset of mycorrhiza formation but fail to complete the development process and do not move on to the next stage. They could be classified into different basic types: hyphal weft not formed; hyphal weft formed but no further development; Hartig net development normal but mantle fails to form. The authors suggest that the mechanisms for the differentiation of the mantle and the Hartig net are independent. It appears that differential gene expression has resulted in finite phenotypic differences between seemingly genetically identical mycelia.

Whatever the genetic basis of these variants, they provide an excellent opportunity for studies of ectomycorrhiza development. Non-mycorrhizal variants, for example, have been used to demonstrate that polypeptide biosynthesis is significantly affected in non-mycorrhizal variants compared to the mycorrhizal ones (Martin, Hilbert, and Kropp, unpublished observations). Whether the polypeptides lacking in the non-mycorrhizal strain are involved in the recognition processes or in mycorrhiza development remains to be demonstrated. This series of *Laccaria* variants provides an indication of the genetic pathway leading to mycorrhiza formation. Sequential action of genes probably occurs up to the adhesion of the symbionts. After primordium formation, the developmental effects of the variants operate independently; the Hartig net can occur without mantle formation, for example[64]. These processes might be regarded as under separate genetic control. The key question of how the genes involved in mycorrhiza formation are turned on and off during development is not yet amenable to experimental studies. However, recent data available on IAA-synthesis in intraspecific variants[17] and UV mutants of *H. cylindrosporum* (Gay and Debaud, unpublished observations) suggest that the regulatory mechanisms operating in the mantle, and Hartig net formation, are under hormonal control. Further research would be greatly aided by the molecular cloning of the genes involved in tryptophan and IAA synthesis (see also review by Gogala in this issue).

Changes in the patterns of polypeptide synthesis during ectomycorrhiza development. Isolation and characterization of developmentally critical genes will not only provide molecular markers for the analysis of mycorrhiza development, but will also allow the basis of their tissue-specific regulation to be investigated. Our analyses have focused on the *Eucalyptus globulus/Pisolithus tinctorius* symbiosis for several reasons: its ontogeny is well characterized[29, 45]; it grows rapidly and is easily manipulated; growth conditions can be defined to give a great degree of experimental reproducibility[39]; and compatible and incompatible variants of *P. tinctorius*[38] are available that can complement biochemical investigations. This eucalypt ectomycorrhizal association is a favorable system that can be used as a paradigm for studying cellular differentiation and morphogenesis using available techniques of molecular biology and genetics. Attempts have been made to divide the development of eucalypt ectomycorrhiza into successive stages[29], namely the 'preinfection', 'infection and mycorrhiza formation', and 'mycorrhiza function' stages. In the preinfection stage, the fungus recognizes its host plant and attaches to the root epidermis, an event that is followed by root hair disappearance. At present nothing is known about specific genes that are involved in this stage, but they may control the microfibrillae described above[38]. In the latter stages, the formation of a knot of interwined hyphae is observed and some hyphae enter the root. In the further stages the formation of the mantle and the Hartig net takes place. In the final stage further differentiation occurs, leading to a physiologically active symbiosis.

Our initial studies on the expression of symbiotic genes have been confined to the stages corresponding to mantle formation and even later stages. Analysis of the mycorrhiza protein pattern by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)[27] revealed that during the differentiation of these ectomycorrhizas the synthesis of proteins is markedly affected. The effects of mycorrhizal infection on polypeptide accumulation fell into three distinct categories: 1) decreased amounts of a large number of polypeptides ('polypeptide cleansing'); 2) increased accumulation of a comparatively small number of polypeptides; and 3) synthesis of mycorrhiza-specific proteins called ectomycorrhizins. Approximately 50% of the fungal polypeptides and more than 80% of the plant polypeptides resolved on gels were affected during the development of ectomycorrhizas. Among 700 polypeptides analyzed on two-dimensional gels, only 10 polypeptides appeared to be specifically synthesized in the symbiosis. Four ectomycorrhizins (E_{10}, E_{11a}, E_{11b}, E_{13b}) gave rise to intense spots when a dense fungal sheath developed. In fully-developed ectomycorrhizas (2 weeks/post-inoculation), E_{24a} and E_{24b} appeared as major spots, whereas they occurred at a low level in week 1, providing evidence for differential accumulation of ectomycorrhizins.

The steps involved in mycorrhiza formation show that major decisions determining the development of an ectomycorrhiza are made at stages preceding the formation of the fungal sheath. For elucidating the possible functions of ectomycorrhizins in this process it is essential to know in which developmental stage ectomycorrhizin genes are expressed. We therefore studied the changes in gene expression from pre-contact to the end of the Hartig net formation. A large decrease in the amounts of numerous polypeptides ('polypeptide cleansing') was evident two days post-inoculation, and a portion of the plant encoded-polypeptides that decreased in concentration may be localized in the root hairs that disappear at this stage. At least three plant genes that encode ectomycorrhizins E_{10b}, E_{16}, and E_{18} were expressed 24 h after inoculation when the initial aggregates of hyphae were formed. These ectomycorrhizins were referred to as early ectomycorrhizins[28]. Analysis of labelled proteins on 2D-PAGE, following in vivo incorporation of [^{35}S]methionine by eucalypt ectomycorrhizas, revealed that a large part of these differences in protein level are the result of differential protein biosynthesis rather than post-translational modifications of the polypeptides[28]. This labelling procedure gives an overview of the expression of the 200 most-abundantly transcribed ectomycorrhiza genes. From the ectomycorrhizins encoded by these genes, 5 give rise to major spots on 2D-PAGE (fig.). The expression pattern of early ectomycorrhizin genes during mycorrhiza development suggests that the products of these genes are involved in mycorrhiza morphogenesis since their appearance accompanies the formation of the symbiosis. Expression of the ectomycorrhizin genes, therefore, coincides with successive steps in the development of the symbiosis, and it will be convenient if one of each group of polypeptides can be used as a molecular marker for a particular stage of development. By turning on the expression of ectomycorrhizin genes and by increasing the expression of symbiosis-stimulated genes, the two symbionts have the ability to raise their assimilating capacity and hence, symbiotic efficiency.



Changes in protein biosynthesis during ectomycorrhiza development. Fluorographs of two-dimensional gels of in vivo [^{35}S]methionine-labelled proteins isolated from non-infected roots of *Eucalyptus globulus* (A), free-living mycelia of *Pisolithus tinctorius* (B), and 2-day-old ectomycorrhizal roots. Samples were incubated in [^{35}S]methionine for 4 h and ^{35}S-labelled polypeptides (approx. 3.0×10^5 cpm TCA-precipitable) were separated by 2D-gel electrophoresis (IEF, SDS-PAGE) and visualized by fluorography of dried gels. The major ectomycorrhiza-specific polypeptides are indicated by →, the ectomycorrhiza-enhanced polypeptides by ▲ and some ectomycorrhiza-reduced spots by ▼. Molecular weights are shown in kDa.

In mycorrhizal fungi, as in all organisms, the plasma membrane probably has two main roles: 1) the physical separation of the inside and outside of the cell with the bidirectional transport of solutes, and 2) sensory transduction, i.e., the sensing and initiation of the cellular response to changing environmental conditions. Fungal and host plasma membranes are thus expected to play a central role in supporting the symbiotic state. Increase in the surface area of the fungal plasmalemma accompanies Hartig net differentiation. Ultrastructural studies[32] indicate that the surface/volume ratio of the fungal cell increases strikingly relative to the ratio in the free-living mycelium, and this requires membrane biogenesis. Although many similarities exist between the plasma membrane of free-living symbionts and ectomycorrhiza/plasma membranes, the mycorrhizal plasmalemma must also possess additional mycorrhiza-specific or mycorrhiza-enhanced polypeptides because of its role in mediating all the molecular exchanges between the host and the microsymbiont. Among the molecules that require active transport via the symbiotic plasma membrane are amino acids, sucrose, and ions. We have, therefore, begun to analyze mycorrhiza-specific polypeptides from mycorrhiza microsomal membranes. Comparative 2D-PAGE of microsomal membranes of ectomycorrhizas, free-living mycelium and uninfected roots confirms the existence of common proteins, and also identifies mycorrhiza-repressed and mycorrhiza-enhanced membrane-bound proteins (Henrion and Martin, unpublished observations). The large number of differences found between the protein profiles of the mycorrhizal and non-mycorrhizal microsomal membranes indicates that the plasma membrane has undergone many modifications to meet the requirements of symbiosis. The large number of metabolite exchanges across the plasma membranes envisaged by current theories makes it likely that some of these mycorrhiza-enhanced proteins will have transport functions, possibly assisted by ATPases.

Differentiation in eucalypt ectomycorrhizas is associated with extensive changes in the concentration of particular polypeptides and in protein biosynthesis. Perhaps the most salient aspects of the work reviewed are that 1) transition from free-living partners to symbionts is accompanied by 'polypeptide cleansing' and 2) ectomycorrhiza formation coincides with a change in the expression of a few mRNAs that produce abundant polypeptides, the ectomycorrhizins. Differential polyadenylation or differential rates of turnover of particular mRNAs cannot yet be distinguished from differential transcription of specific DNA sequences. Extensive turnover of mRNAs and 'polypeptide cleansing' have been associated with developmental processes such as meiosis[51]. Such degradative changes in eucalypt ectomycorrhizas appear to occur during the initiation of the plectenchymatous sheath[28]. Differential transcription, including expression of tissue-specific genes, has been extensively studied in other higher fungi[4, 51, 63] and in roots interacting with microorganisms[8, 61]. It will be of interest to determine to what extent changes in morphogenetic complexity and polypeptide biosynthesis in the two symbionts are associated with changes in gene transcription. cDNA libraries of free-living partners and of ectomycorrhizal tissues have been developed, and the cloning of genes which are differentially expressed during the formation of eucalypt ectomycorrhiza is underway (Martin and Hilbert, unpublished results). Further studies should reveal the timing of expression and the regulation of these 'symbiotic genes', while their isolation and sequence analysis may provide clues as to mechanism of their regulation and their function.

Developmental regulation of ectomycorrhizas – a proposal

From the work described here and from studies of developmental regulation in other filamentous fungi[4, 51, 63], it is possible to construct a working model describing genetic and biochemical changes that accompany ectomycorrhiza initiation and the development of symbiosis. Major alterations in patterns of gene expression occur during, and are required for, ectomycorrhiza development. The basic regulatory mechanism probably involves hierarchies of nuclear gene expression. At the top level would be a set of genes, similar to the *brlA* gene of *Aspergillus nidulans*[1], whose activity mediates the developmental switch from the indeterminate, apical growth pattern of vegetative mycelium to the highly organized pattern of the ectomycorrhiza. The activity of these genes is necessary and sufficient to induce a regulatory cascade that is partially responsible for ectomycorrhizal ontogeny. These genes may respond to rhizospheric signals and positional information mediated by 'sensory molecules'. These genes probably activate the mycorrhiza development program, and 'reinforce' decisions during most phases of ectomycorrhiza ontogeny. At lower levels in the regulatory hierarchy are genes that regulate the details of symbiosis development, which are likely to differ among mycorrhizal species and host-trees.

Concluding remarks and future research

During its development, the ectomycorrhizal symbiosis undergoes a series of differentiation events leading to a new, integrated organ. Ultrastructural observations combined with cytochemical and biochemical studies reveal that biochemical and structural changes in the symbiont cells lead to the final phenotype of the active ectomycorrhiza. With the present state of our knowledge of the developmental biology of ectomycorrhizas, it is only possible to speculate about genetic events during development. A number of steps controlling mycorrhiza formation have been characterized, and gene expression has been emphasized in this review at the expense of environmental aspects of development. This reflects the paucity of data available on the environmental stimuli that trigger development, and on associated biochemical events.

A large number of genes are required to program the entire process of mycorrhiza development. Recent increased interest in the molecular processes has resulted in the isolation of mycorrhiza-modulated polypeptides. These ectomycorrhizins are now being characterized and rapid progress in this work can be expected. Determining the function of the mycorrhiza-specific polypeptides and their associated genes in ectomycorrhiza development is a major goal for the future. Regarding their abundance, these ectomycorrhizins could be tentatively identified as structural or metabolic polypeptides that might be involved in major functions. A greater challenge will be to identify those genes ('primary regulators') that are critical in initiating the unique developmental events during ectomycorrhiza differentiation. In general, well-defined mutants blocked for each step of the mycorrhiza formation are keys to understanding the formidable complexity of this developmental process.

As pointed out by Reijnders and Moore[55], "There is a finite number of ways of modifying the structure of a hyphal cell during differentiation, but there may be an infinite number of ways in which those differentiated cells can be assembled into different structures". For example, the development of any organized fungal structure – ectomycorrhizal sheath or fruit body – requires that hyphae grow towards one another and cooperate in the formation of the differentiating organ. Vegetative and sexual structures impose similar developmental requirements on the mycelium and it will be interesting to compare genes involved in the initiation of fruit body development with those controlling the first events of ectomycorrhiza differentiation.

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Regulation of mycorrhizal infection by hormonal factors produced by hosts and fungi

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Summary. An overview of current research on hormonal factors produced by plants and fungi in mycorrhizal associations is presented. On the one hand, growth hormones in roots and their exudates influence the metabolism and growth of fungi. On the other, fungal hormones influence root morphology, metabolic changes and the growth of the entire plant.

Key words. Mycorrhiza; plant hormones; fungi; root exudate; roots; growth substances.

Introduction

There are few topics in plant physiology and ecology with as many open questions as research on mycorrhizas. The one in focus here concerns the regulation of mycorrhizal infection, and in particular the role of plant hormones. According to Allen[1], the formation of mycorrhizas represents a special adaptation to surviving in unsuitable conditions and under stress in the habitat. In general, adaptations of plants to changes in the external environment involve changes in plant growth and development and are controlled by plant hormones[114]. It is no wonder, therefore, that the role of plant hormones has been a focus of attention of investigators of mycorrhizas since the discovery of the auxins. Later, after the discovery of other growth regulators, their presence in mycorrhizal