MOLECULAR AND CELLULAR ASPECTS OF THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS

Maria J. Harrison
The Samuel Roberts Noble Foundation, Ardmore, Oklahoma 73402; e-mail: mjharrison@noble.org

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ABSTRACT
Arbuscular mycorrhizae are symbiotic associations formed between a wide range of plant species including angiosperms, gymnosperms, pteridophytes, and some bryophytes, and a limited range of fungi belonging to a single order, the Glomales. The symbiosis develops in the plant roots where the fungus colonizes the apoplast and cells of the cortex to access carbon supplied by the plant. The fungal contribution to the symbiosis is complex, but a major aspect includes the transfer of mineral nutrients, particularly phosphate from the soil to the plant. Development of this highly compatible association requires the coordinate molecular and cellular differentiation of both symbionts to form specialized interfaces over which bi-directional nutrient transfer occurs. Recent insights into the molecular events underlying these aspects of the symbiosis are discussed.

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INTRODUCTION
The symbiotic associations of plant roots and fungi have intrigued many generations of biologists, and in the late 1880s these associations were given the name mycorrhiza—derived from the Greek for fungus-root (68). Recent observations of fossil plants from the Devonian era suggest that one type of mycorrhizal association, the arbuscular mycorrhiza, existed approximately 400 million years ago (MYA), indicating that plants have formed associations with arbuscular mycorrhizal (AM) fungi since they first colonized land (143, 151). Today, the arbuscular mycorrhiza is the most widespread type of mycorrhizal association and exists in ecosystems throughout the world where it creates an intimate link between plants and the rhizosphere (6, 95, 180, 190). Despite the ubiquitous occurrence of this association and its importance in nutrient movement between plants and the soil, our understanding of the mechanisms underlying the development and functioning of the symbiosis is limited.

This review builds on the information from two earlier Annual Reviews of the arbuscular mycorrhizal symbiosis, in which the physiology and regulation of the symbiosis were discussed (110, 178). In the conclusions to their 1988 review concerning the physiological interactions between the symbionts (178), Smith & Gianinazzi-Pearson noted a need for approaches to provide fundamental information at a molecular level about the development and functioning of the association. Over the past ten years there has been an explosion of molecular, genetic, and biochemical analyses of the AM fungi and the AM symbiosis. This review emphasizes insights into aspects of the development of the symbiosis emerging from these studies. It focuses briefly on the AM fungi and the advances in our understanding of these obligate symbionts and then on aspects of development of the association, and finally briefly considers the mechanisms underlying nutrient transport, which are still almost entirely unknown.

ARBUSCULAR MYCORRHIZAL FUNGI
The arbuscular mycorrhizal fungi are all members of the zygomycota and the current classification contains one order, the Glomales, encompassing six genera into which 149 species have been classified (27, 129). A major factor
hampering studies of the AM fungi, including the taxonomy, is their obligately biotrophic nature; so far, they have not been cultured in the absence of a plant host. Despite the lack of axenic culture, it is possible to grow them in sterile culture with plant roots, or with so-called hairy roots transformed with *Agrobacterium rhizogenes* (23, 56, 130, 132). Recent adaptations of these methods utilize petri plates in which the fungus and root are cultured together in one compartment while the external mycelium is permitted to ramify into a second compartment separate from the roots (185). Increasing numbers of fungal species are being established in this culture system (54, 57, 60), which is proving useful for studies of the fungal symbiont (12, 13, 25, 47, 106, 116). Such systems also provide access to pure, sterile fungal spores and mycelium that are essential for molecular analyses and useful for the taxonomy of these fungi.

**Molecular Analyses of the Fungal Genome**

There has been one report of sexual structures in AM fungi, but it remains unconfirmed. Recent genetic analyses also suggest that the fungi are asexual and reproduce clonally (153). The large resting spores formed by these fungi are unusual in that they are multinucleate and, depending on the species, may contain thousands of nuclei per spore (24). The nuclei in quiescent spores are arrested in the G0/G1 phase (29) and although initial studies suggested that DNA replication did not take place without a plant host (40), this was later demonstrated to be incorrect; both DNA replication and nuclear division occur during the initial growth of the hyphal germ tube, regardless of the presence or absence of a host plant (24). Using nuclear stains and flow cytometry, the DNA content of the nuclei has been estimated to range from 0.13 to 1.0 pg per nucleus for 12 species tested (30, 103). Analysis of the base composition of nine different species of glomalean fungi, containing representatives from four different genera, indicated that the genomes of these fungi have a relatively low GC content, averaging 33%, and in contrast to other fungal taxa, a high level of methylcytosine (2.23–4.26%), although not as high as that of plant genomes (102).

The first AM fungal genes to be sequenced were the small subunit rRNA genes (SSU) and the internal transcribed spacer (ITS) regions that were targets for phylogenetic analyses (119, 149, 169, 170). From the SSU sequence data it was possible to estimate a date for the origin of the AM fungi and the time at which further divergence within the group occurred. The origin of the AM fungi was placed between 462 and 353 MYA and the ancestral fungi were probably most like the extant Glomus species. The families Acaulosporaceae and Gigasporaceae emerged later and were estimated to have diverged from each other 250 MYA (170).

The SSU and ITS sequence data also allowed the design of specific primers that, when coupled with PCR amplification, enabled the identification of AM
fungi from both spores and within plant roots in field situations (49, 55, 119, 149, 159, 160, 170, 171). Additional DNA-based methods of identification followed (120, 195, 209), including random amplification of polymorphic DNA (RAPD) analysis. This enabled the development of primer-pairs specific for a number of species including *Glomus mosseae*, *Gigaspora margarita*, and *Scutellospora castanea* (1, 114, 206). These primers have utility in taxonomic studies and in competitive PCR assays to quantify the amount of fungus within mycorrhizal roots (59, 66).

A particularly interesting finding that emerged during the molecular analyses of the ITS regions was the variability in the genetic composition within and between spores of a single fungal species. A single spore may contain more than one ITS sequence and individual spores have ITS sequences that differ from other spores of the same species (119, 159). This variability was further confirmed by analyses of other loci (153, 210). Using minisatellite-based markers, it was observed that the first generation of spores arising from single-spore cultures displayed a high level of variation, which suggests that the multinucleate spores are heterokaryotic. It seems likely that reassortment of genetically different nuclei provides a mechanism by which these fungi maintain genetic diversity (210).

Genomic libraries prepared from spores and cDNA libraries from spores and mycorrhizal roots have been constructed for a limited number of species (69, 192, 208), and the first cDNA clones representing genes other than the rRNAs have been identified. Glyceroldehyde-3-phosphate dehydrogenase (GAPDH), β-tubulin, ATPase, nitrate reductase, and DNA-binding protein sequences were among the first to be reported. Although most of these are considered housekeeping genes, they will be useful molecular markers for analysis of these fungi during the development of the symbiosis (42, 69, 70a, 107).

Future dissection of gene function in arbuscular mycorrhizal fungi will be difficult without the possibility to transform these fungi genetically. Progress toward this goal is being made and transient expression of a reporter gene construct in spores of *Gigaspora margarita* has been achieved. This in itself is an important technological achievement and will enhance molecular analyses of the AM fungi (67).

DEVELOPMENT OF THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS

Early Signaling Events
Germination of AM fungal spores and the initial growth of hyphal germ tubes can occur in the absence of the plant root; however, both root exudates and
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Volatiles such as CO₂ can stimulate both of these processes (15, 22, 25, 47, 84, 108, 135, 144, 152). In some cases, root exudates also elicit rapid and extensive branching of the hyphae as they enter the vicinity of the root (89, 91), a response that has been observed as hyphae approach the roots of host plants but not when they encounter non-host roots, which suggests that recognition of the host occurs. In this case, lack of recognition of the non-host could be due to lack of a signal; however, in other instances non-host status is probably due to inhibitory compounds (164, 165, 199).

The range of active components present in root exudates is unknown. How-ever, some of the activities are probably due to flavonoid and phenolic compounds that stimulate the growth of some AM fungal species while inhibiting others (22, 62, 144, 172, 191). The particular molecule(s) responsible for eliciting hyphal branching is also unknown but, based on its estimated size, it could also be a phenolic or flavonoid derivative (90). Since the flavonoid compounds are active at very low concentrations, it is assumed that they do not have a nutritional effect but rather that they act as signals to stimulate or inhibit growth. Plant flavonoid/iso flavonoids bind to estrogen receptors, and recent experiments utilizing estrogens and anti-estrogens provide preliminary evidence for the presence of an AM fungal receptor capable of binding biochanin A and estrogens. Based on the structures of these molecules, it is suggested that the A and C rings of the isoflavonoid and the hydroxyl group at position A-7 are important features for recognition by the receptor (145). Although flavonoid derivatives can influence the initial stages of the fungal life cycle, experiments with flavonoid-deficient mutants of maize indicate that they are not essential for the development of the symbiosis (26). Maybe in natural environments, the flavonoid-mediated stimulation of growth and branching in the vicinity of the root helps to ensure contact with the root and the establishment of the symbiosis. The differential effects of flavonoids/iso flavonoids on different fungal species could be envisaged to influence the fungal populations associated with particular plants.

**Appressorium Formation**

Development of the symbiosis is initiated when a fungal hypha contacts the root of a host plant where it differentiates to form an appressorium. Although components of root exudates are capable of stimulating hyphal growth and branching, they are unable to elicit the formation of appressoria, which were initially only observed on intact plant roots (88). Recently, it was demonstrated that Gigaspora margarita could form appressoria in vitro on purified epidermal cell walls isolated from carrot roots, a host for Gigaspora margarita, but not on walls isolated from sugar beet, a non-host (134). The fungus also recognized specifically the epidermal cell walls and did not form appressoria on cortical or
vascular cell walls. These experiments indicate that the signal for appressorium formation lies within the epidermal cell wall, a hypothesis suggested earlier by Tester et al (188). The experiments also confirm that the branching signal is either loosely bound to the wall or exuded from the roots, since the purified wall fragments did not elicit the extensive hyphal branching observed in intact roots. These purified walls probably consist of a mixture of polysaccharides, including cellulases and polygalacturonans and some proteins. Carbohydrate molecules act as signals in a number of other plant/fungal interactions and are likely candidates for the induction of appressoria in the AM symbiosis (121).

**Penetration of the Root**

Appressorium formation is followed by the development of a penetration hypha and penetration of the root. This can occur in different ways; in some species, the hypha enters by forcing its way between two epidermal cells, whereas in other cases, the hypha penetrates an epidermal or root hair cell wall and grows through the cell (34). The exact mechanisms involved in penetration are unknown; however, by analogy to a number of the biotrophic pathogens (127), it has been suggested that specific, localized production of cell wall–degrading enzymes, in combination with mechanical force, may facilitate entry of the hyphae without inducing defense responses (33). AM fungi produce exo- and endoglucanases, cellulases, xylanases, and pectolytic enzymes including polygalacturonase (76–78, 80, 150), all of which would expedite their passage through a cell wall.

Since the appressoria that developed on the purified epidermal cell wall fragments failed to form a viable penetration hypha and did not penetrate the wall, processes subsequent to appressorium formation likely require an intact cell (134). A wide range of plant mutants on which the AM fungi can form appressoria but cannot develop further are direct proof that the plant controls this developmental step in the association. Mutants blocked at this stage of this symbiosis have been described in *Pisum sativum* (63, 86), *Medicago sativa* (37, 38), *Vicia faba* (63), *Phaseolus vulgaris* (168), *Medicago truncatula* (155), *Lotus japonicus* (203), and *Lycopersicon esculentum* (21). The phenotypes of these mutant associations are fairly similar at the morphological level and fall into two broadly defined groups. In association with the *P. sativum, L. esculentum,* and *Medicago* mutants, the fungus forms appressoria that are frequently large and deformed and that become septate when the fungus fails to enter the root (21, 37, 86). In one of the *Medicago sativa* mutant lines, the number of appressoria formed on the mutant increases, a possible consequence of the failure to penetrate (37); however, increased numbers of appressoria were not reported for the *P. sativum* mutants (86). In *P. sativum,* the non-penetrating phenotype is referred to as myc(−1) and 21 such mutants have been described.
They belong to five complementation groups, which indicates that entry into the root is under complex genetic control. The traits segregate as single recessive loci and the condition is determined by the roots (86). Grafting wild-type scions onto mutant stocks did not rescue the mutants (86, 200). Hairy roots prepared from these genotypes also maintain their nonmycorrhizal status (14). Cytochemical analyses of one of the *P. sativum* and one of the *M. sativa* mutant interactions indicated that cell wall depositions, including callose and phenolics, were present in the walls of cells adjacent to the appressoria (93, 142). Such depositions were not seen in wild-type interactions, which suggests that a defense response has been elicited in these mutants. Based on these data, it is possible that a suppressor of defense responses has been mutated such that the plant now views the fungus as a pathogen. This situation is reminiscent of the barley/Erysiphe graminis interaction where mutation-induced recessive alleles of the *Mlo* locus confer resistance to a wide range of isolates of *Erysiphe* (71). Resistance is mediated by the formation of appositions on the cell walls below the appressoria and wild-type *Mlo* is a negative regulator of defense responses as well as leaf cell death (71). The *Mlo* gene has been cloned and the encoded protein is predicted to be an integral membrane protein; however, the function of the protein and mechanism of regulation of defense responses remain to be determined (43).

The *P. vulgaris* and *L. japonicus* mutants show a slightly different phenotype from the other species and appressorium formation is followed by penetration of the first cell layer (203). The association then aborts in the root epidermis where swollen and deformed hyphae are visible within these cells (203). In the *Lotus* mutants, the hyphae occasionally manage to overcome the block in the epidermis and growth from the deformed hyphae continues, producing normal internal mycorrhizal structures. Since these are indistinguishable from wild-type, it has been suggested that the mutated genes are not required for the later phases of growth (203). All of the legume mycorrhizal mutants are also affected in their ability to form a nitrogen-fixing symbiosis with *Rhizobium* species and thus define a set of genes, termed *sym* genes, essential for both symbioses. Similarities between these symbioses are just beginning to emerge, and some of the signaling pathways and downstream events occurring during the formation of the symbioses clearly are conserved (2, 194).

A nonpenetrating mutant identified in *L. esculentum* is the first mutant of this type to be identified from a nonleguminous species. This mutant shows a similar phenotype to the legume mutants, although slightly different responses were noted depending on the fungal symbiont involved. *Glomus mosseae* was unable to penetrate the *L. esculentum* mutant roots, whereas *Gigaspora margarita* was occasionally able to enter. In contrast to the *L. japonicus* mutants, this mutation appears to affect the internal stages of development of the mycorrhiza, and
following entry, *G. margarita* did not develop extensively within the roots and was unable to form arbuscules (21). The future cloning of the mutated genes, which should be feasible for *L. esculentum* and also for the legumes, *L. japonicus* and *M. truncatula*, due to their small genome size, will provide insight into the controlling mechanisms.

**Internal Growth and Development of Arbuscules in an Arum-Type Mycorrhiza**

Following entry into the root, internal development of the fungus is influenced by the plant, and a single species of fungus may show significantly different morphological growth patterns depending on the plant partner in the association (82, 105). The two main patterns are referred to as the *Paris* and *Arum* types, named after the species in which they were first described (74, 174). Much of the laboratory research focuses on crop species that form the *Arum* type and molecular investigations of the *Paris* type have not been undertaken. Thus the *Arum* type is the focus of these discussions.

In the *Arum*-type mycorrhiza, penetration of the root is initially followed by intercellular hyphal growth, although in some instances the fungus will penetrate the exodermis and form hyphal coils in the exodermal cells as it passes through (34). On reaching the inner cortex, branches arising from the intercellular hyphae penetrate the cortical cell walls and differentiate terminally within the cell to form dichotomously branched structures known as arbuscules (Figure 1). Although an arbuscule develops within a cell, it remains essentially apoplastic as the plant plasma membrane extends to completely surround it. The fungal cell wall becomes progressively thinner as the arbuscule develops and consequently in these cells, there is an extensive intracellular interface in which the two symbionts are in extremely close contact, separated only by their membranes and a narrow plant-derived apoplast (35, 36, 178). This interface is thought to be the site at which phosphate and possibly carbon are transferred between symbionts, although some speculate that the intercellular hyphae might be responsible for carbon uptake (173, 176, 181). Despite the intensive effort expended by both symbionts to develop the arbuscule and the arbuscular interface, the life span of an arbuscule is only a few days, after which it collapses and decays leaving the cell undamaged and capable of hosting another arbuscule (4, 5). Both the variable growth patterns observed in the *Arum*- and *Paris*-type hosts and a *P. sativum* myc (−2) mutant in which the arbuscules barely develop, indicate that the plant also controls this stage of the association (83, 86).

Following formation of arbuscules, some species of AM fungi also form lipid-filled vesicles within the roots, which are presumed to act as a storage reserve for the fungus (178).
Figure 1  Diagram indicating possible sites of plant and fungal phosphate transporters and fungal glucose transporters in membranes of an arbuscular mycorrhiza (Arum type). Phosphate transport: 1, phosphate uptake across membranes in the external hyphae; 2, phosphate efflux across the arbuscule membrane; 3, phosphate uptake across the peri-arbuscular membrane. Carbon transport: 2 and 4, possible sites of glucose uptake by the fungus.

MOLECULAR AND CELLULAR ALTERATIONS IN THE CELLS DURING ARBUSCULE DEVELOPMENT  As the fungal hypha penetrates a cortical cell wall and begins to differentiate into an arbuscule, the invaded cell responds with fragmentation of the vacuole, migration of the nucleus to a central position within the cell, and an increase in the number of organelles (17, 33, 46). This response seems to be arbuscule specific and it does not occur in the exodermal cells during the development of coils. The plasma membrane extends approximately fourfold to form the peri-arbuscular membrane (5) that envelops the arbuscule, and therefore concomitant increases in membrane biosynthesis must be occurring. Although the peri-arbuscular membrane is connected to the peripheral membrane of the plant cell, cytochemical analyses indicate that it has high levels of ATPase
activity, whereas the remainder of the plasma membrane shows little staining for ATPases (87). Since H\(^+\)-ATPase activity gives rise to the proton gradient required for many secondary active transporter processes, these data support the suggestion that active transport of nutrients may be occurring across this membrane. Currently, little else is known about activities associated with the peri-arbuscular membrane and the fact that it develops deep within the tissues of the root makes it recalcitrant to many of the modern techniques of membrane biology. It is assumed to retain the ability to synthesize and deposit cell wall components including \(\beta\)-1,4 glucans since these have been found in the new apoplastic compartment formed between the peri-arbuscular membrane and the arbuscule (33). Immunocytochemical analyses have also demonstrated the presence of a matrix composed of pectins, xyloglucans, nonesterified polygalacturonans, arabinogalactans, and hydroxyproline-rich glycoproteins (HRGP) in this compartment (20, 33, 92, 140a). Consistent with this composition, genes encoding both a putative arabinogalactan protein (AGP) and an HRGP have been shown to be induced in mycorrhizal roots of \textit{M. truncatula} and maize, respectively, and the transcripts are localized specifically in the cells containing arbuscules (19, 193).

Although the interface compartment is continuous with the cell wall and the molecular content tends to reflect the composition of the cortical cell wall (18), the physical structure is considerably different. The components of the interface matrix do not become cross-linked and most closely resemble primary cell walls (33). The lack of cross-linking has been suggested to be the result of lytic enzymes released from the arbuscule, and in support of this hypothesis, endopolygalacturonase has been immunolocalized to the arbuscule and interface compartment (33, 139). In pea mycorrhizae, immunocytochemical analysis of the interface matrix indicates the presence of components in common with the peribacteroid compartment of pea nodule cells. These epitopes are not present in the peripheral cell walls, providing further evidence for the specialization of the interface matrices and reiterating similarities between the two symbioses (140a, 141). Whether the components of the arbuscular interface matrix have any symbiosis-specific functions is unclear; however, AGP-like proteins have been reported in two other symbiotic associations. They are induced both during nodule development in the \textit{Rhizobium}-legume symbiosis (163) and also in the \textit{Gunnera/Nostoc} association, where they are abundant in the \textit{Gunnera} stem gland mucilage, which plays a central role in communication between the symbionts (147). In plant/biotrophic fungal pathogen associations, it was shown recently that the fungus produces a proline-rich glycoprotein that is very similar to the plant cell wall proline-rich and hydroxyproline-rich glycoproteins. The protein is deposited in the extrahaustorial matrix, a component analogous to the arbuscular interface matrix, where it is suggested to mimic plant cell wall proteins.
and prevent the plant from perceiving the pathogen (140). Components of the arbuscular interface matrix might conceivably function in a similar manner.

The specific changes associated with formation of the arbuscular interface clearly require significant reorganization of the cytoskeleton of the cell. Recent confocal microscopy studies confirm this hypothesis and demonstrate the presence of new cortical microtubule networks running along the hyphae and also between the hyphae and the nucleus of the cortical cell. This latter observation is consistent with earlier studies documenting the movement of the nucleus of the colonized cortical cell to a central position adjacent to the arbuscule (17, 81). A maize α-tubulin promoter is also activated specifically in cells in which arbuscules are developing, providing further evidence of an increase in cytoskeletal components in these cells (32).

It is predicted that development of the arbuscular interface will be accompanied by many other alterations in the cortical cells, and documentation of these changes is just beginning. In M. truncatula, transcripts encoding enzymes of the flavonoid biosynthetic pathway, phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), but not the defense-specific enzyme isoflavone reductase (IFR), are induced specifically in cells containing arbuscules (98). Since secondary metabolites from this pathway accumulate in mycorrhizal M. truncatula roots, the location of expression of these two genes has led to the speculation that they might be synthesized in these cells (97, 98). The potential function of the flavonoids/iso flavonoids within these cells is unclear; however, some of them stimulate growth of AM fungi both in the presymbiotic phase of their life cycle and during the symbiosis (47, 191, 207). In other cases, flavonoids act as auxin transport inhibitors (104) and therefore alter the hormone balance in the roots. Hormone levels are known to change in mycorrhizal roots and are probably responsible for the induction of expression of some of the mycorrhiza-induced genes (194). Transcription of an auxin-inducible gene encoding glutathione S-transferase (GST) is also induced in cells containing arbuscules (186). GSTs are stress-response enzymes that add glutathione (GSH) to a variety of compounds including phenylpropanoids/flavonoids to facilitate their transport via a specific carrier into the vacuole (53, 124). Sequestration in the vacuole prevents phytotoxic effects on the cell that can occur if high levels of these compounds accumulate. Although in this case the flavonoid and GST experiments were performed in different plants, it is not surprising that they are co-induced in the same cell type.

The External Mycelium

Following colonization of the root cortex, fungal hyphae develop extensively within the soil. This external mycelium plays a pivotal role in the AM symbiosis where its functions include the acquisition of mineral nutrients from the soil and
their subsequent translocation to the plant, colonization of additional roots, and, in many cases, the production of spores. In addition to its role in the symbiosis, the extraradical mycelia contributes to soil stability by the aggregation of soil particles, probably mediated in part by glycoproteins produced by the hyphae (204, 205).

Studies of this phase of the symbiosis have lagged behind that of the internal phase, mainly owing to difficulties with observation, collection, and quantification of the mycelium. However, new methods that overcome many of these problems are now available (137, 185). Early studies of the external mycelium had indicated that it is comprised of different types of hyphae, including large runner hyphae and finer absorptive hyphae (72). Recently, these findings have been confirmed, and an ultrastructural examination of the fine absorptive hyphae has revealed features consistent with a role in nutrient absorption (10, 11). Measurements of both the internal pH of the hyphae and the external pH of the media surrounding the hyphae are also possible in this system; therefore, the physiological state of the hyphae can be monitored. (13, 106). Since appropriate tools are now available, studies of this phase of the symbiosis should be emphasized in the future.

IDENTIFICATION OF GENES INDUCED DURING THE ARBUSCULAR MYCORRHIZAL SYMBIOSIS

Over the past few years, a search for genes and proteins induced in mycorrhizal roots has been initiated in a number of laboratories. Analysis of protein profiles, differential screening (133, 187, 193), and differential display (125, 126) have all been used to provide insight into molecular changes occurring in both the plant and fungal symbionts during development of the symbiosis. The initial comparisons of the protein profiles of noncolonized and colonized roots indicated the presence of new proteins in mycorrhizal roots but did not permit differentiation between fungal proteins and newly induced plant proteins (8, 65, 79, 158, 162). These differences are more readily resolved via molecular analyses where comparison of cDNA and genomic sequences can be used to determine the genome of origin.

Consistent with the increased synthesis of plasma membrane in the mycorrhizal roots, many of the mycorrhiza-induced plant cDNAs identified so far encode membrane proteins. Differential screening of a cDNA library prepared from barley mycorrhizal roots resulted in the identification of a barley cDNA sharing sequence identity with a H+\textsuperscript{+}-ATPase (133). This clone hybridizes to transcripts that are induced in mycorrhizal roots and although the cell and membrane location of the protein are still unknown, it is a potential candidate for the gene encoding the ATPase located on the peri-arbuscular membrane. A cDNA
encoding a member of the membrane intrinsic protein (MIP) family is induced in mycorrhizal parsley roots. These integral membrane proteins facilitate the movement of small molecules across membranes and might be predicted to have a role in transport at the arbuscular interface (154). Differential display analyses of mycorrhizal pea roots resulted in the identification of a cDNA psam1, predicted to encode a novel protein sharing some similarities with phospholamban, a membrane-anchored protein that regulates the activity of Ca\textsuperscript{2+}-ATPase (125). The function of the psam1 gene product in mycorrhizae is unknown.

Since the development of the matrix at the arbuscular interface requires the de novo synthesis of cell wall proteins, it might also be predicted that this class of proteins would be induced in mycorrhizal roots. Consistent with this hypothesis, genes encoding an HRGP, a putative AGP, and a member of the xyloglucan endo-transglycosylase (XET) family are all induced during development of the association (193). XETs are enzymes that cleave and reform xyloglucan bonds within the cell walls, and it is speculated that this activity might be employed to loosen cell walls to permit penetration of the fungal hyphae or, alternatively, to function in maintaining the structure of the arbuscular interface matrix (193).

In addition to the growing numbers of genes up-regulated during the symbiosis, there are clearly groups of genes that are specifically down-regulated in the mycorrhiza. In Medicago truncatula, it was observed that four phosphate starvation-inducible genes including two phosphate transporter genes, a gene of unknown function (Mt4), and an acid phosphatase homolog were all down-regulated following colonization of the root by a mycorrhizal fungus (41, 118). Elevated levels of phosphate in the external medium down-regulate the expression of these genes, and since the symbiosis generally results in an increased level of phosphate in the roots, the fact that these genes are down-regulated in the symbiosis is not so surprising (41, 118). However, studies of the Mt4 gene indicate that down-regulation of this transcript also occurs in one of the Medicago mutants in which the fungus fails to penetrate the root and grows only on the external surface. In this association, it seems unlikely that the fungus delivers phosphate to the root. Therefore, this suggests that down-regulation of the Mt4 gene can occur also via a signal from the fungus and is consistent with the presence of two, initially independent pathways for the regulation of the Mt4 gene (41).

Although the identification of genes differentially regulated in mycorrhizal roots provides initial information about the changes that occur during development of the mycorrhiza, real insight into mechanisms underlying its development will occur in the future when the roles of these proteins in the symbiosis are elucidated. New technologies, such as confocal laser scanning microscopy coupled with the use of fluorescent reporter proteins such as green fluorescent protein (GFP) permit the in vivo monitoring of gene and protein expression.
within living cells and provide a powerful addition to the current arsenal of molecular tools with which to analyze the symbiosis.

**Expression of Defense Responses**

In many plant-fungal pathogen interactions, invasion of plant tissues by the fungus results in the induction and sustained expression of a varied battery of plant defenses that prevent further pathogen ingress (58). This is not the case, however, with some of the biotrophic pathogens that form ostensibly compatible but parasitic associations with their hosts and probably avoid eliciting defenses by the conservative and local production of hydrolytic wall-degrading enzymes and minimal damage to the plant cell (122, 127, 128). The arbuscular mycorrhiza seems to be the most highly attuned plant-fungal association. Data from many AM associations indicate that in the AM symbiosis plant defense responses generally show small transient increases in the early stages of the symbiosis, followed by suppression to levels well below those of noncolonized plants (85, 109).

In *Allium porrum*, chitinase and cell wall–bound peroxidase activities showed a transient increase in expression during the initial stages of development of the mycorrhiza; however, activity in a well-established mycorrhizal association was notably lower than in the controls (183, 184). In addition, immunocytochemical analysis suggested that the chitinase, when present, was located in the vacuole and was not in contact with the hyphae (183). Similar observations were later made in bean and tobacco roots where colonization by an AM fungus was accompanied by a transient increase and then a decrease in both the transcript levels and activity of chitinase, β-1,3 endoglucanase, and chalcone isomerase, an enzyme of isoflavonoid biosynthesis (52, 112, 197, 201). These data indicated that regulation was occurring probably at the level of gene expression, a finding that is also supported by studies of the expression of genes encoding enzymes of the isoflavonoid biosynthetic pathway in *Medicago* species. *Medicago sativa* and *M. truncatula* respond to attack by fungal pathogens with the rapid induction of genes and enzymes of the isoflavonoid pathway, which results in the production of the defense compound, medicarpin. In contrast, colonization by AM fungi results in the down-regulation of the gene encoding isoflavone reductase (IFR), the penultimate enzyme of medicarpin biosynthesis, and medicarpin does not accumulate (97, 202). In situ hybridization revealed that down-regulation of the IFR transcript occurred exclusively in the areas of the root in which arbuscules had formed, indicating that this is a specific and local effect (98). Investigations of soybean roots colonized with different strains of *G. intraradices* suggested that the suppression of endochitinase expression was correlated with the infectivity of the different strains, with the most infective strains resulting in the most down-regulation (113). This finding might
explain the results from parsley and bean roots where defense responses were not altered following colonization by mycorrhizal fungi (31, 70).

Although the suppression of plant defense responses seems to be a widespread occurrence in AM associations, the necessity for this suppression is challenged by data indicating that *Nicotiana tabacum* and *Nicotiana sylvestris* plants over-expressing various chitinases, glucanases or pathogenesis-related (PR) proteins were apparently entirely unaffected in their ability to form mycorrhizae (196, 198). In contrast, overexpression of these defense proteins inhibits the growth of fungal pathogens. Plants overexpressing chitinase were more resistant to the root pathogen *Rhizoctonia solani*, whereas those overexpressing PR-1a were more resistant to *Peronospora tabacina* and *Phytophthora parasitica* (3, 39, 198). The only gene whose overexpression was observed to inhibit colonization of plants by AM fungi was PR-2, a protein with β-1,3 glucanase activity (196). Although the data from these transgenic plants cast doubts on the requirement for suppression of these particular defense responses, there are other instances in which defense responses seem to be impeding the interaction. In *Salsola kali*, which is a non-mycotrophic species, AM fungi colonize the roots and initially form coils and arbuscules; however, the root responds very quickly with the production of autofluorescent compounds and the colonized areas of the root turn brown and die (7). It might be argued that this is a non-host response; however, similar responses are seen in alfalfa (a mycorrhizal species) in response to colonization by *G. margarita*. The fungus enters the root, but colonized cells show a hypersensitive response and become necrotic. Phenolic and isoflavonoid compounds characteristic of a defense response accumulate in these regions of the root (61). Thus, if defense responses are elicited in the appropriate cells, they can apparently prevent development of the association. These results also indicate that the AM fungi do not simply fail to elicit defense responses, as has been occasionally suggested, but rather that at some level, there is compatibility between the fungus and the plant, and recognition of compatibility prevents induction of defense responses. Incompatible combinations clearly exist, and as different genera and species of fungi are utilized for laboratory experiments, it might be predicted that more of the incompatible interactions will be identified. Additional evidence supporting the hypothesis that recognition and suppression of defense responses is required for successful colonization is provided by the pea locus *a myc* mutant in which defenses seem to be induced and prevent further development of the appressorium (93).

Although in general a sustained defense response is not induced during a successful AM symbiosis, there are some exceptions. For example, the defense gene, PR-1, is expressed in pea root cells containing arbuscules, and in a number of plants new symbiosis-related forms of chitinase are induced in the roots following colonization by mycorrhizal fungi (51, 64, 146). In these instances, the
proteins could have roles other than classical defense. Chitinases released from spruce cells do not have deleterious effects on ectomycorrhizal fungi, but actually destroy fungal elicitors released from fungal cell walls by cleaving them into smaller, inactive units. In this way, the elicitation of defense responses can be prevented and development of the symbiosis can proceed (157). The new chitinase isoforms induced in arbuscular mycorrhizal roots may have a similar role.

Expression of Nodulation Genes in the Mycorrhizal Symbiosis

The emerging similarities between the Rhizobium-legume symbiosis and the arbuscular mycorrhizal symbiosis have stimulated investigations of the expression of nodulation genes during the AM symbiosis. Leghemoglobin transcripts were detected in mycorrhizal roots of Vicia faba (73), whereas in Medicago sativa, two nodulation genes, MsENOD40 and MsENOD2, were induced in mycorrhizal roots with similar tissue-specific patterns of expression as in roots inoculated with Rhizobium (194). Both genes can be induced in roots in the absence of a symbiosis via the application of cytokinin, and since cytokinin levels are elevated during nodulation and also in mycorrhizal roots (194), it is speculated that cytokinin is one component of the signal transduction pathway mediating induction of these genes during the symbioses. Further evidence of signal transduction pathways common to both symbioses is provided by studies of the PsENOD5 and PsENOD12A genes, which are induced in pea roots during interactions with either AM fungi or Rhizobium. In the pea sym8 mutant, which is unable to form either of these symbioses, expression of both genes is blocked, suggesting that SYM8 functions in the signal transduction pathway for induction of these genes in both symbioses (2). Based on these studies and also on the legume symbiosis mutants, it is clear that some mechanisms are shared between the two symbioses and this has fuelled speculation that the Rhizobium-legume symbiosis arose by exploiting signaling pathways from arbuscular mycorrhizae (194). Since our understanding of the Rhizobium-legume symbiosis is more advanced than that of the arbuscular mycorrhizal symbiosis, it will be fruitful to exploit these overlaps to elucidate these aspects of the association.

NUTRIENT TRANSPORT ACROSS ARBUSCULAR MYCORRHIZAL INTERFACES

Nutrient transport between the symbionts is a central aspect of the symbiosis; however, the membrane transporters responsible for the movement of carbon or phosphate between the symbionts are unknown (Figure 1). In addition, there is speculation as to the interfaces involved in carbon transport between the plant
and the fungus. [For in depth discussions of these issues, readers are referred to a number of comprehensive reviews (173, 179, 181).] A brief summary of the current opinions on transport in the symbiosis and insights obtained from recent data that will facilitate identification of the transport proteins are included here.

**Carbon Transfer from the Plant to the Fungus**

Although it has been known for over 20 years that carbon is transferred from the plant to the fungus, evidence as to the form of carbon has been lacking (28, 101). Recent in vivo $^{13}$C nuclear magnetic resonance spectroscopy data strongly suggest that glucose is the form of carbon utilized by these fungi (167) and this is further supported by studies of isolated arbuscules that were observed recently to use glucose for respiration (182). Although carbon allocation to the roots increases during mycorrhizal associations, the amounts of carbon estimated to leak out of intact root cells into the apoplast are thought to be insufficient to account for the amount of fungal growth occurring in mycorrhizal roots. Therefore, enhanced efflux, or a decrease in the level of competing host uptake systems, has been proposed (138, 166, 173, 181). In *M. truncatula*, expression of a hexose transporter gene is induced in mycorrhizal roots, specifically in the cortical cells in the vicinity of the fungus, which suggests that in this case potentially competing host mechanisms are not suppressed (96). In other symbioses, enhanced efflux of nutrients, stimulated by the demand of the microsymbiont, has been observed and in some plant/fungal pathogen interactions, the fungi produce toxins that alter membrane transport processes to favor release of metabolites (75, 100, 123). Similar events may well occur during the AM symbiosis, and the fungal symbiont may possess the capability to stimulate efflux of carbon from the plant. So far, there is no molecular information about transport proteins responsible for the efflux of carbon out of plant cells; however, this is currently an active area of research since this type of transporter is also expected to exist in the mesophyll and vascular tissues where sugar export occurs (161).

Although the arbuscule presents a large area of close contact between the symbionts and was traditionally assumed to be the interface over which carbon would be transferred, the observation that the membrane of the arbuscule lacks ATPase activity has led to the suggestion that carbon uptake might occur via the intercellular hyphae, whose membranes have been observed to have a high ATPase activity and thus are energized for active transport processes (87, 176) (Figure 1). It is unclear whether uptake of carbon by the AM fungus requires an active transport mechanism similar to those of plant transporters, or whether concentrations of carbon at the interfaces could be sufficient to permit uptake by facilitated diffusion, as occurs in yeast (111). In the absence of information about the concentrations of carbon present in the various apoplastic interfaces, it
is difficult to speculate on potential mechanisms of transport either out of plant cells or into the fungus. *Amanita muscaria*, an ectomycorrhizal fungus, utilizes both fructose and glucose but relies on plant invertases for release of these hexoses from sucrose (48, 156). A monosaccharide transporter was cloned recently from *Amanita muscaria* and is probably the transporter responsible for hexose uptake in both the free-living and symbiotic stages of its life (136). A similar transporter might be envisaged for AM fungi, although it seems unlikely that it will be present in the absence of the plant host. Sequence information from the *Amanita* transporter coupled with those from yeast and *Neurospora crassa* may facilitate the cloning of transporters from arbuscular mycorrhizal fungi.

**Phosphate Transfer from Soil to the Plant via the Fungus**

Phosphate movement in the symbiosis involves a number of membrane transport steps, beginning with uptake across membranes in the external hyphae. This is followed by translocation back to the internal fungal structures where it is thought to be released from the fungus across the arbuscule membrane and then taken up into the plant by transporters on the peri-arbuscular membrane (Figure 1) (173, 178, 181). There has been some progress toward understanding the mechanisms of uptake of phosphate by the external AM fungal hyphae and a high-affinity phosphate transporter has been cloned from *G. versiforme* (99). The transporter has a $K_m$ of 18 $\mu$M as determined by expression in yeast cells, a value that is consistent with previous measurements of phosphate uptake by AM fungi (189). The transporter transcripts are present in the external mycelium and not in the structures of the fungus internal to the root and therefore the transporter may be responsible for the initial uptake of phosphate into the mycorrhiza (99). Unfortunately, the inability to perform gene disruption experiments in arbuscular mycorrhizal fungi currently prevents direct proof of its role in the symbiosis.

Phosphate flux across the symbiotic interfaces in the mycorrhiza has been estimated at 13 nmol m$^{-2}$s$^{-1}$, although this value increases if extra phosphate is supplied to the mycorrhiza (50, 177). In contrast, the general rate of efflux of phosphate from fungal hyphae growing in culture was measured at 12 pmol m$^{-2}$s$^{-1}$ (45). Based on these findings, the AM fungi likely have some type of specialized efflux mechanism operating in the arbuscule membrane to permit sufficient phosphate efflux to the arbuscular interface. Efflux of phosphate from hyphae of an ectomycorrhizal fungus was shown to be stimulated by divalent cations, and a similar mechanism, possibly triggered by a component of the interface matrix, might be envisaged for the AM fungi (45). Since the peri-arbuscular membrane has a high ATPase activity (87), the subsequent uptake of phosphate by the plant could then occur by proton-coupled transport.
mechanisms. However, as with the previous discussion of carbon transport, the lack of information about physiological conditions at the arbuscular apoplastic interface prevents informed speculation as to the type of transport mechanisms. Analysis of the conditions in the apoplastic interfaces in mycorrhizal roots is extremely challenging, although progress is being made (9).

In the past two years, phosphate transporters have been cloned from the roots of a number of plant species (115, 117, 118, 131, 175). These transporters are expressed during growth in low-phosphate environments and mediate high-affinity phosphate transport into the epidermal and cortical cells. In *Medicago truncatula*, the expression of these transporters is down-regulated in mycorrhizal roots (118). This suggests that the plant does not use these transporters during the symbiosis and therefore it seems unlikely that these transporters operate at the peri-arbuscular membrane. Studies of phosphate uptake in mycorrhizae have revealed that in some associations, phosphate uptake directly via the root cells was considerably reduced during the symbiosis and the bulk of the phosphate uptake occurred via the fungal symbiont (138a), which is consistent with the down-regulation of the root phosphate transporters during the symbiosis.

The cloning and functional analysis of the transport proteins operating in the symbiosis is just one of the challenges for the future. Part of this process might be accelerated by utilizing approaches that have been successful in other research fields, such as the plant/biotrophic fungal pathogen associations, where there have been recent advances in the identification of transporters operating in the haustorial membranes. The cloning of an amino acid transporter from a rust fungus was achieved from libraries prepared from isolated haustoria (94). With the refinements in methods for isolation of arbuscules, similar approaches might permit access to the fungal transport proteins operating at the arbuscule membrane.

**CONCLUSIONS**

Over the past few years, the first insights into molecular mechanisms underlying development of the symbiosis have been achieved. The initial similarities to, and differences from, other plant-microbe interactions have been explored and exploited. Despite this progress, there is a still a vast amount to learn, and the molecular approaches that have been initiated recently need to be integrated with other disciplines to address both development and functioning of the symbiosis.

Although the current range of mycorrhizal mutants is limited, they have played a pivotal role in proving that the plant controls various stages of development of the association. The complexity of this symbiosis renders genetic approaches essential, and the identification of additional mutants, particularly
those that are specific for the mycorrhizal symbiosis, should be emphasized in the future.

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