Arbuscular Mycorrhizal Fungi Elicit a Novel Intracellular Apparatus in *Medicago truncatula* Root Epidermal Cells before Infection

Andrea Genre, a Mireille Chabaud, b Ton Timmers, b Paola Bonfante, a and David G. Barker a–1

a Department of Plant Biology, University of Turin and Istituto per la Protezione delle Piante–Consiglio Nazionale delle Richerche, 10125 Turin, Italy
b Laboratory of Plant–Microbe Interactions, Unité Mixte de Recherche Institut National de la Recherche Agronomique–Centre National de la Recherche Scientifique, 31326 Castanet Tolosan Cedex, France

The penetration of arbuscular mycorrhizal (AM) fungi through the outermost root tissues of the host plant is a critical step in root colonization, ultimately leading to the establishment of this ecologically important endosymbiotic association. To evaluate the role played by the host plant during AM infection, we have studied in vivo cellular dynamics within *Medicago truncatula* root epidermal cells using green fluorescent protein labeling of both the plant cytoskeleton and the endoplasmic reticulum. Targeting roots with *Gigaspora* hyphae has revealed that, before infection, the epidermal cell assembles a transient intracellular structure with a novel cytoskeletal organization. Real-time monitoring suggests that this structure, designated the prepenetration apparatus (PPA), plays a central role in the elaboration of the apoplastic interface compartment through which the fungus grows when it penetrates the cell lumen. The importance of the PPA is underlined by the fact that *M. truncatula dmi* (for doesn’t make infections) mutants fail to assemble this structure. Furthermore, PPA formation in the epidermis can be correlated with *DMI*‐dependent transcriptional activation of the *Medicago* early nodulin gene *ENOD11*. These findings demonstrate how the host plant prepares and organizes AM infection of the root, and both the plant–fungal signaling mechanisms involved and the mechanistic parallels with *Rhizobium* infection in legume root hairs are discussed.

Arbuscular mycorrhizae (AM) are highly specialized endosymbiotic associations formed between a restricted group of biotrophic soil fungi (the Glomeromycota) and the large majority of vascular land plants, including most angiosperm and gymnosperm families. Fossil evidence shows that AM symbiosis has existed for >450 million years (Remy et al., 1994), and this unique beneficial fungal–plant association is believed to have played a major role in the early colonization of land plants. AM fungi penetrate and colonize the root, forming highly differentiated symbiotic structures known as arbuscules, which are the principal sites of metabolic exchange between the two organisms (reviewed in Harrison, 2005). Concomitant extraradical hyphal development allows the fungus to supply important nutrients, including phosphate, to the host, while in return receiving carbohydrates from the plant. The AM symbiosis also confers resistance to the plant against biotic and abiotic stresses.

Despite the agronomic and ecological importance of the AM symbiosis, the molecular and cellular events associated with the establishment of the association are poorly understood. This is primarily attributable to the difficulty in culturing these obligate fungi, coupled with the low frequency and lack of synchrony of host infection. Nevertheless, it is now clearly established that, before infection, germinated AM fungi respond to host root exudates by switching to an active presymbiotic growth phase, which leads to intense hyphal ramification (or branching) in the vicinity of the root (Giovannetti et al., 1993; Buée et al., 2000). Very recently, it was shown that the active molecules in host root exudates responsible for this characteristic branching response are sesquiterpene lactones (Akiyama et al., 2005). After activation, hyphae make contact with the root epidermis and continue ramifying, with concomitant differentiation of surface appressoria. Infection hyphae then develop from appressoria and penetrate outer root tissues. Cytological studies have shown that intracellular AM infection hyphae that traverse epidermal cells are enclosed within an apoplastic compartment of plant origin, comprising a plasmalemma invagination and associated matrix (Novero et al., 2002). This initial step in root colonization is then followed by extensive intraradical hyphal development, with associated arbuscule formation in the inner cortex, as well as by extraradical development and subsequent spore formation.

To date, remarkably little is known about the crucial stage of the interaction that follows the initial fungal–plant contact and precedes infection, and in particular the nature of the molecular/cellular dialog that is required for recognition of the fungal partner and successful infection. However, genetic studies performed with several legume genera, such as *Pisum, Medicago*, and *Lotus*, have revealed that a small group of plant genes are

1 To whom correspondence should be addressed. E-mail barker@toulouse.inra.fr; fax 33-561-285061.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: David G. Barker (barker@toulouse.inra.fr).

Online version contains Web-only data.

Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.105.035410.
essential for successful root penetration (reviewed in Parniske, 2004). These genes were originally identified by virtue of their role in early steps of Rhizobium-elicited nodulation, and in particular in transducing the specific rhizobial symbiotic signal (Nod factor) perceived by root hairs, essential for bacterial infection (reviewed in Limpens and Bisseling, 2003). In the case of the model legume Medicago truncatula, mutations in three distinct genes (DOESN’T MAKE INFECTIONS1 [DMI1], DMI2, and DMI3) result in a block of root infection by either Sinorhizobium meliloti or AM fungi (Catoira et al., 2000). For the AM fungal association, surface recognition and appressorium formation are observed for all dmi mutants, but there is a total or partial block of epidermal penetration (Catoira et al., 2000; Morandi et al., 2005). The role of the DMI genes in Nod factor signaling has led to the proposition that AM fungi generate analogous Myc signals, whose perception is required to initiate infection (Albrecht et al., 1999). In addition to these genetic data, molecular studies in a variety of legumes have revealed that a number of host genes expressed early during nodulation, including ENOD2, ENOD5, ENOD11, ENOD12, and ENOD40, are also transcribed during root colonization by AM fungi (van Rhijn et al., 1997; Albrecht et al., 1998; Journet et al., 2001).

To facilitate detailed molecular/cellular studies of the AM infection process, an experimental system was recently developed for M. truncatula using Agrobacterium rhizogenes–transformed roots targeted with negative geotropic Gigaspora germination hyphae (Chabaud et al., 2002). Such transformed root cultures can be successfully colonized by a whole range of AM fungi, and importantly, root cultures derived from dmi mutants retain their infection-defective symbiotic phenotypes (Chabaud et al., 2002; Kosuta et al., 2003). By means of a reporter gene strategy, Chabaud et al. (2002) exploited this system to demonstrate that the M. truncatula early nodulation gene, ENOD11, transcribed during rhizobial infection (Journet et al., 2001; Boisson-Dernier et al., 2005) is also expressed specifically in root epidermal and cortical cells directly associated with root infection by AM fungi. Furthermore, the absence of ENOD11 expression in appressorium-contacted epidermal root cells derived from a dmi2 mutant suggests that the DMI-dependent signaling pathway is required for infection-related gene activation.

Despite the genetic and molecular analogies that can be made between Rhizobium and AM infection, it is still unclear how and to what extent the plant plays an active role in the AM penetration process (Parniske, 2000). To attempt to answer these questions, we made use of the AM-targeting root culture system described above, in conjunction with green fluorescent protein (GFP)-tagged markers, to monitor intracellular dynamics in the host epidermis throughout AM infection. This approach has revealed that, before infection, a nucleus-directed cytoskeletal/endooplasmic reticulum (ER) apparatus is assembled within the epidermal cell in response to appressorium formation. This transient assembly, which we have designated the prepenetration apparatus (PPA), defines the subsequent path of hyphal infection and is most likely responsible for synthesizing the apoplastic compartment required for hyphal containment. Parallel experiments with dmi mutants have confirmed the importance of the PPA in the infection process, and a cellular GFP tag driven by the ENOD11 promoter has been used as a marker to study the relationship between PPA formation and fungal-plant signaling. The discovery of this major intracellular restructuring preceding fungal entry reveals how the host plant responds to and accommodates the AM fungal symbiont and provides an important cellular framework to comprehend the nature of controlled endosymbiotic infection.

RESULTS

GFP-Labeled Cellular Markers Expressed in Root Tissues of M. truncatula

M. truncatula root clones expressing appropriate GFP-labeled markers for monitoring both the plant cytoskeleton and the ER were generated to study the in vivo intracellular responses and remodeling that occur during the preinfection and infection stages of the AM–plant association (see Methods). GFP:Map4-MBD (Marc et al., 1998) was chosen to visualize the microtubular cytoskeleton, GFP:Fimbrin1-ABD (Voigt et al., 2005) was chosen to label actin filaments, and GFP-HDEL (Haseloff et al., 1997) was chosen for ER labeling. The localization of the three GFP-based fusions was first evaluated in epidermal cells of control non-colonized roots. Figure 1A illustrates the typical parallel, predominantly oblique arrays of cortical microtubules labeled by the GFP:Map4-MBD fusion protein (Marc et al., 1998), and Figure 1B shows GFP:Fimbrin1-ABD labeling of the bundled actin microfilaments that originate in the perinuclear cytoplasm and spread across the cell. Finally, the GFP-HDEL marker labels the characteristic ER structure, comprising a lace-like network of lamellar and tubular cisternae present throughout the cortical cytoplasm, with a maximum density around the nucleus (Figure 1C). As expected, time-lapse imaging (data not shown) revealed very dynamic structures for both the fine cortical network of actin filaments (Voigt et al., 2005) and the ER (Staehein, 1997). We conclude that the transgenic M. truncatula root clones expressing these three GFP cellular tags can be used to identify and follow changes in the structure of the epidermal cell cytoskeleton and ER as well as movements of the cell nucleus in the case of the actin and ER tags.

Epidermal AM Infection Is Preceded by Nucleus-Directed Assembly of a Novel Cytoskeletal/ER Apparatus

For the studies described in this article, the targeted AM inoculation technique developed for in vitro–cultured M. truncatula roots (Chabaud et al., 2002) was modified to permit continuous microscopic observation (see Methods). Microscopic analysis of >100 independent infection events using the three GFP markers and two Gigaspora species (with time-lapse imaging for a number of penetration events) led to the identification of a series of host cellular responses that are systematically associated with epidermal AM fungal infection. These responses, described in detail below, can be subdivided into those that precede fungal penetration and those that are associated with subsequent intracellular hyphal growth. In particular, the epidermal cell nucleus, with two distinct phases of intracellular movement, appears to play a central role in the dynamics associated with the preinfection responses, which include the assembly of a novel cytoskeleton/ER-containing structure.
Figure 1. Intracellular Dynamics in the Wild-Type *M. truncatula* Root Epidermis throughout AM Infection.

(A) to (C) Control roots expressing cytoskeletal/ER GFP tags. (A) Parallel cortical arrays (arrows) of microtubules labeled with GFP:Map4-MBD. (B) Actin microfilament bundles radiating from the perinuclear actin cage (arrows) labeled with GFP:Fimbrin1-ABD. Note that weak autofluorescence of cell walls is visible in red. (C) A cortical lace-like network of lamellar and tubular ER cisternae (arrows) labeled with GFP-HDEL, with a maximum density around the nuclei. All images are z axis projections of serial optical sections. n, nucleus. Bars ¼ 20 µm.

(D) to (I) Fungal contact and appressorium formation (autofluorescence of *G. gigantea* in red) elicits rapid nuclear movement to the ACS and a number of intracellular rearrangements that occur before the subsequent transcellular nuclear migration. (D) Initial nuclear movement toward the ACS is accompanied by the reorganization of microtubules into a network of randomly oriented bundles (arrows). Once in the vicinity of the ACS, microfilament bundles (arrow) are formed between the nucleus and the ACS (E) and large ER patches accumulate below the ACS (F). Note that the indicated positions of the epidermal cell nuclei in (E) and (F) are based on both GFP tagging and the corresponding transmitted light images (not shown). Subsequently, dense subconical microtubules (arrow) are formed below the ACS (G) as well as radial arrays of microfilament bundles (arrow) (H) and a doughnut-like structuring of ER patches (arrow) (I). All images are z axis projections of serial optical sections. n, nucleus; arrowheads, approximate position of the ACS. Bars ¼ 20 µm.
AM root infection initiates from surface appressoria, whose formation is generally indicated by tip growth arrest and associated hyphal swelling. Observation of numerous hyphal contacts with associated appressoria formation suggests that the epidermal cell nucleus rapidly moves toward and positions itself directly below the site of appressorium contact (or ACS). Several time-lapse observations have shown that this initial nuclear movement is generally completed within <2 h. Associated with this first phase of nuclear movement, we have also observed cortical microtubules reorganizing from parallel loop arrays to a network of randomly oriented bundles (Figure 1D). Note that the nuclear position is visible only with the actin and ER GFP tags.

During this nuclear repositioning at the ACS and before the initiation of the second phase of nuclear migration, we have observed thick actin bundles radiating from the nucleus toward the ACS (Figure 1E) and large patches of ER assembly below the ACS and around the nucleus (Figure 1F). This is followed by additional cytoskeletal/ER restructuring, which includes the assembly of a dense subconical set of microtubules (Figure 1G) as well as a radial array of actin bundles (Figure 1H). The ER patches assemble into an approximately doughnut-shaped configuration below the ACS (Figure 1I) as the nucleus initiates a migration across the cell lumen away from the appressorium at an estimated speed of 15 to 20 μm/h. This migration is accompanied by the creation of a broad cytoplasmic column linking the nucleus to its initial position below the ACS. Within this column, all three GFP markers reveal the assembly of a striking structure comprising a high-density array of microtubules (Figure 1J), microfilament bundles running parallel to the column (Figure 1K), and a very dense region of ER cisternae (Figure 1L). Three-dimensional imaging further reveals that the dense ER structure is in fact a hollow tube joining the nucleus to the ACS (see Supplemental Figures 1A to 1D online). Estimations from time-lapse imaging suggest that 4 to 5 h are required for the formation of this novel intracellular structure after initial fungal contact and appressorium formation.

**Hyphal Penetration Follows the Path Defined by the Transcellular Nuclear Migration**

Our confocal observations have shown that fungal penetration occurs precisely at the site of initial cytoplasmic aggregation associated with nuclear positioning below the ACS. This is illustrated by comparing Figure 1G (subconical microtubule arrays at the ACS) with Figure 1M, which shows the identical site 13 h later after successful fungal penetration. Furthermore, our observations suggest that hyphal penetration only initiates once the cytoplasmic column has totally traversed the cell lumen, and in all cases subsequent fungal growth strictly follows the transcellular path laid down by the cytoskeleton/ER-containing structure formed within the column (see the time-lapse images in Figures 1P to 1R and associated Supplemental Video 1 online). We have calculated from such time-lapse studies that hyphal growth across the lumen (often running diagonally across the length of the epidermal cell) is completed in ~3 h, with an estimated speed of 20 μm/h. Finally, the hyphal penetration event depicted in Figures 1P to 1R and in Supplemental Video 1 online shows clearly that the GFP-labeled ER tube is progressively widened as the hypha traverses the cell.

The images shown in Figures 1M to 1O illustrate hyphae that have penetrated, and in certain cases completely traversed, the epidermal cell layer. Once hyphae have crossed the cell, the nucleus is in general no longer positioned at the end of the cytoplasmic column (clearly visible in Figure 1O). This suggests that the nucleus detaches from the end of the column and repositions at the cell periphery once infection has been completed. Although cytoskeletal/ER labeling is still present peripheral to the penetrating hyphae (Figures 1M to 1O), the overall fluorescence intensity of the GFP markers has decreased significantly compared with that observed before infection (cf. with Figures 1J to 1L). We interpret this finding as a dismantling of the preinfection assembly at some stage after hyphal entry. In our experiments, hyphal penetration has been observed both through the outer epidermal cell wall and through the anticlinal wall between adjacent epidermal cells, as described for *Lotus japonicus* infection (Bonfante et al., 2000).

To provide further evidence for a direct relationship between the formation of the cytoskeleton/ER-containing structure and the synthesis of the perifungal membrane, we stained the root with the vital lipophilic fluorescent dye FM 4-64 (Bolte et al., 2004), which labels the epidermal plasma membrane in control roots (Figure 2A). Figure 2B shows that FM 4-64 labels a putative membrane invagination with a shape and position consistent with the cytoplasmic column and its associated preinfection

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**Figure 1.** (continued).

(J) to (L) Major intracellular restructuring is observed within the broad cytoplasmic column created between the nucleus and the ACS during the transcellular nuclear migration. (J) A dense array of microtubules (arrows). (K) Parallel bundles of microfilaments (arrow). (L) A very dense region of ER cisternae (arrow). Note that in (L) the nucleus is positioned against the rear wall and has fully traversed the cell. All images are z axis projections of serial optical sections. n, nucleus; arrowheads, approximate position of the ACS. Bars = 20 μm.

(M) to (O) Fungal penetration of the epidermal cell occurs precisely at the site of initial cytoplasmic aggregation. Although at a lower level of fluorescence, microtubule arrays (M), microfilament bundles (N), and ER (O) are still visible around the penetrating hypha. Note that the image in (M) shows successful fungal infection at the identical site illustrated in (G). In (O), the nucleus is no longer positioned at the end of the cytoplasmic column. All images are z axis projections of serial optical sections. n, nucleus; double arrowheads, penetration site. Bars = 20 μm.

(P) to (R) Time-lapse images of fungal penetration through the cytoplasmic column and toward the nucleus, showing the widening of the GFP-HDEL–labeled ER (arrows) around the hypha as it progresses across the cell. Note that before fungal penetration (P), the nucleus is positioned against the rear wall and has fully traversed the cell. A complete animation is presented in Supplemental Video 1 online. All images are z axis projections of serial optical sections. n, nucleus; arrowhead, approximate position of the ACS; double arrowhead, penetration site. Bars = 20 μm.
structure. Such membrane invaginations were never observed in control stained roots. Additional FM 4-64 labeling experiments using GFP-HDEL–expressing roots further showed that the FM 4-64 staining colocalizes with the PPA and is present before fungal entry (see Supplemental Figures 1E to 1G online).

Together, our results show that the formation of a specific structure comprising microtubules, microfilaments, and ER is preceded by the migrating nucleus within a cytoplasmic column traversing the epidermal cell, and this structure plays a key role in constructing the apoplastic compartment through which the

Figure 2. Evidence for Interface Membrane Formation and ENOD11 Gene Expression in the Epidermis of Wild-Type Roots before AM Infection and Limited Intracellular Responses in Infection-Defective dmi Roots.

(A) to (C) Vital staining of M. truncatula root tissues with the lipophilic dye FM 4-64 (green). (A) Noncolonized control epidermal cells show staining of the plasma membrane (arrow). (B) Appressorium formed by AM fungal hypha (red) on the root surface is associated with an FM 4-64–stained putative membrane invagination (arrow). The arrowhead indicates the future hyphal penetration site. (C) Absence of membrane invagination associated with potential appressorium (arrowhead) in dmi2-2 roots. All images are z axis projections of serial optical sections, and three-dimensional imaging (not shown) clearly reveals that in (C) the fungus is on the outer root surface. Bars = 20 μm.

(D) to (F) Evidence for nuclear migration toward surface fungal hyphae is presented for both dmi2-2 [(D] and [E]) and dmi 3-1 (F), visualized by GFP tagging of either microfilaments (D) or ER ([E] and [F]). All images are z axis projections of serial optical sections. n, nucleus. Bars = 20 μm.

(G) to (I) Wild-type roots expressing the P<sub>ENOD11</sub>:GFP-HDEL construction. (G) ER-associated fluorescence is present predominantly in epidermal cells contacted by fungal appressoria (arrowheads), but without discernible PPA structures. (H) Intense fluorescent labeling of ER cisternae (arrow) associated with a characteristic PPA connecting the nucleus to the future penetration site (arrowhead). (I) Epidermal ER fluorescence is weak after fungal penetration (arrows). All images are z axis projections of serial optical sections. The double arrowhead marks the penetration site. n, nucleus. Bars = 20 μm.

(J) to (M) These four stills represent progressive stages leading to AM infection deduced from our studies and taken from the animated movie shown in Supplemental Video 2 online. Appressorium formation on the outer surface of the host cell (J) results in initial nuclear movement toward the surface appressorium (K). This is followed by the assembly of the transient PPA within the cytoplasmic column created during the subsequent transcellular nuclear migration (L). Finally, the AM infection hypha crosses the epidermal cell through the apoplastic compartment constructed within the cytoplasmic column (M). Color coding (which differs slightly compared with the animation) is as follows: cell nucleus, dark brown; plasma membrane, light brown; microtubules; green; actin bundles, red; ER, white.
fungal infection hyphae subsequently traverse the epidermal layer. We have designated this novel transient structure the PPA.

**PPAs Are Not Formed in Epidermal Cells of Roots Derived from dmi2 and dmi3 Mutants**

As mentioned previously, mutations in any of the three DMI genes of *M. truncatula* result in plants that are totally or partially defective in AM fungal infection of the root (Catoira et al., 2000; Morandi et al., 2005), and we have previously shown that the mutant phenotype is conserved in *A. rizogenes*-transformed roots (Chabaud et al., 2002; Kosuta et al., 2003). To examine the extent to which the *dmi* root epidermis responds to the AM fungus, we introduced the GFP:Fimbrin1-ABD and GFP-HDEL fusions into roots of *dmi2* (*dmi2*-2 allele) and *dmi3* (*dmi3*-1 allele) mutants (see Methods) and established high-level GFP-expressing root cultures for each construct.

When transgenic roots derived from the two *dmi* mutants were targeted with germination hyphae of either *Gigaspora gigantea* or *Gigaspora rosea*, the hyphae ramified in the vicinity of the roots and fungal appressoria were formed on the root surface, as described previously for *dmi2* (Chabaud et al., 2002). Throughout the entire experimental period (1 to 2 weeks), neither *Gigaspora* species was able to colonize the *dmi2/dmi3* mutant roots; indeed, not a single penetration event was observed among at least 80 fungal–root contacts examined. These inoculation conditions clearly differ from those of Morandi et al. (2005), who recently reported that the Myc<sup>−</sup> phenotype of the *dmi2*-2 mutant can be partially overcome after a lengthy 2- to 4-week coculture with the virulent AM fungus *Glamus intraradices*.

Significantly, detailed microscopic analyses of AM fungal–root contacts, making use of both ER and actin markers, failed to reveal any cellular responses for either *dmi* mutant indicative of the formation of a PPA structure (such as the assembly of ER cisternae). Likewise, plasma membrane invaginations in epidermal cells underneath ramifying fungal hyphae were never observed in labeling experiments with the vital FM 4-64 marker (Figure 2C). On the other hand, we did observe a frequent positioning of epidermal cell nuclei directly below ramifying hyphae on the *dmi2* and *dmi3* root surfaces (Figures 2D to 2F). Together, these results suggest that the epidermal cells in roots of *dmi2* and *dmi3* are still capable of responding to fungal contact with an initial phase of nuclear movement toward the contact site, but there is a subsequent defect in their capacity to initiate PPA formation.

**Correlation between PPA Formation and the Transcriptional Activation of the *M. truncatula* ENOD11 Gene**

The *M. truncatula* ENOD11 gene encodes a repetitive Pro-rich protein, believed to be a functional component of the plant extracellular matrix (Journet et al., 2001). As stated previously, studies with transgenic *M. truncatula* expressing a *P<sub>ENOD11</sub>:GUS* reporter fusion showed that gene expression is activated in epidermal/cortical cells associated specifically with AM fungal infection, and this activation is absent in a *dmi2* mutant background (Chabaud et al., 2002). To investigate the relationship between the transcriptional activation of ENOD11 and PPA formation, the ENOD11 promoter was fused to the GFP-HDEL reporter (see Methods). After introduction of this construct into wild-type *M. truncatula* roots, gene activation in the root epidermis before and during AM infection was followed by means of the ER-localized fluorescent label.

*Gigaspora*-targeting experiments systematically revealed *P<sub>ENOD11</sub>:GFP-HDEL* activation in epidermal cells in contact with fungal appressoria (Figure 2G) as well as in a very limited number of adjacent cells. Note that there is little if any fluorescence induction in the majority of the surrounding epidermal cells, suggesting that ENOD11 activation is probably cell autonomous within the epidermal layer. Among the fluorescent epidermal cells examined, a few showed typical PPA structures associated with very intense ER labeling. Figure 2H illustrates one such cell in which the fungus has not yet entered the cell. At a later stage of infection, when the fungus has traversed the epidermal cell, ER fluorescence around the fungus is still visible, but at a significantly lower level (Figure 2I). This finding is in agreement with the results obtained earlier with the 35S-driven GFP-HDEL construct (cf. with Figures 1L and 1O).

In conclusion, these observations suggest that the *M. truncatula* ENOD11 gene is activated in epidermal cells after fungal appressorium differentiation both before and during PPA formation and probably also during hyphal penetration. It also appears that not all of the cells expressing *P<sub>ENOD11</sub>:GFP-HDEL* are subsequently infected. Because our time-lapse experiments have shown that ~4 to 5 h are required for PPA formation after initial fungal contact, we deduce that the earliest ENOD11 gene activation presumably occurs within the first hours after appressorium formation.

**DISCUSSION**

**The PPA, a Novel Plant Intracellular Structure Preparing AM Root Infection**

In this article, we have described in detail the cellular responses elicited in the host plant root associated with the preparation of the epidermal cell layer for penetration by AM fungi. Real-time imaging coupled with GFP tagging of cytoskeletal/ER components has revealed a complex multistep host response that precedes fungal entry and that ultimately leads to the synthesis of the transcellular apoplastic compartment separating the penetrating hypha from the host cytoplasm. Most striking among our findings has been the identification of a novel structure comprising microtubules, microfilaments, and ER, which we termed the PPA. This structure is assembled within a column of cytoplasm created during the progressive migration of the nucleus across the epidermal cell and defines the future path taken by the infection hyphae. The fact that the identical structure is labeled by all three fluorescent markers makes it extremely unlikely that the PPA is an artifact resulting from the use of transgenic roots expressing GFP tags. The reasons for proposing the PPA as a key cellular actor in AM infection are based on the following observations. (1) The formation of the PPA is strictly associated with the transcellular nuclear migration that always initiates from a position directly below the appressorium (Figures 1J to 1L). (2) Successful hyphal infection always follows...
the transcellular path laid down by the PPA (Figures 1P to 1R). (3) PPA formation and the associated nuclear migration are totally absent in roots derived from the two infection-defective dmi mutants (dmi2-2 and dmi3-1) that we have analyzed. (4) Expression of the infection-related ENOD11 gene can be observed in PPA-containing cells (Figure 2H).

Together, these findings lead us to propose that the PPA is a major player in the synthesis of the membrane–matrix interface that surrounds and isolates the infection hypha from the cell cytoplasm. This is supported by the labeling experiments using the vital stain FM 4-64, which revealed a putative membrane structure that colocalizes with the cytoplasmic column and associated PPA (Figure 2B; see Supplemental Figures 1E to 1G online). Additional experiments are now required to study the dynamics of membrane synthesis and the role of other cellular components required for membrane–matrix synthesis, such as the Golgi apparatus. We estimate from the observation of numerous AM infections that 7 to 8 h are required for both PPA formation and hyphal crossing of the epidermis. This relatively short time, coupled with the transient nature of the PPA and the overall low frequency of infections, probably explains why this transient structure has not been observed previously in chemically fixed tissues. The discovery of the PPA suggests that the plant root plays a central role in preparing for and directing AM infection, and the implications of this in terms of cellular signaling between the plant and fungal symbiont are discussed below. An animated movie, presenting the entire AM infection process, in particular the roles of the plant cell nucleus, cytoskeleton, and ER in PPA formation, can be viewed in Supplemental Video 2 online. Four stills illustrating key steps from the animation are presented in Figures 2J to 2M.

At this stage, it is too early to conclude that the intracellular infection mechanism that we have observed for M. truncatula interacting with both G. gigantea and G. rosea can be generalized for all types of AM fungi and for all host plants. Interestingly, studies with the model legume L. japonicus have shown that Nod+ Myc- mutants such as Ljsym4-2 are blocked for hyphal infection within the epidermal layer (Wegel et al., 1998) rather than before epidermal entry, as for Medicago. One possible interpretation of this difference could be that, in the case of the Lotus mutants, fungal hyphae penetrate the epidermis despite the fact that the plant cell has failed to prepare for infection by synthesizing the PPA. Such a failure would presumably result in the observed aborted infection and associated host cell death (Bonfante et al., 2000; Genre and Bonfante, 2002). Therefore, it will be of considerable interest to examine host cellular dynamics in Lotus wild type and infection-defective mutants.

**Symbiotic Fungal–Plant Signaling Leading to PPA Synthesis**

We have shown that two distinct phases of nuclear movement within the epidermal cell precede and accompany PPA assembly (presented schematically in Figure 3; see also Figures 2J to 2M and Supplemental Video 2 online). The first phase is presumably triggered by AM hyphal contact and appressorium formation and leads to a positioning of the nucleus directly below the ACS. At present, we do not know whether this nuclear repositioning is a specific response to AM fungi. However, because we still observe nuclear movement toward sites of fungal contact for dmi2/dmi3 mutants, it is probable that this early step does not require signaling through a DMI gene–dependent pathway. By contrast, the PPA assembly and the associated transcellular nuclear migration are clearly dependent on functional DMI2 and DMI3 genes. DMI2 is a receptor-like kinase with a leucine-rich repeat–containing extracellular domain (Endre et al., 2002), and DMI3 is a putative calcium- and calmodulin-dependent kinase (Lévy et al., 2004; Mtra et al., 2004). Because dmi7 mutants have a similar Myc- phenotype to dmi2 and dmi3 (Catrin et al., 2000), it is highly likely that inactivation of the third DMI gene (encoding a putative membrane ion channel [Ané et al., 2004] will also lead to a block in PPA synthesis. As stated previously, the fact that the three DMI genes are required for initiating root infection in both rhizobial and AM symbioses, and more specifically for transducing the rhizobial Nod factor signal, has led to the suggestion that AM fungi produce an analogous so-called Myc factor (Albrecht et al., 2004).
Our findings imply that a crucial step essential for fungal entry occurs after hyphal contact and appressorium formation, but before PPA assembly. Therefore, we hypothesize that the generation of a Myc signal at the appressorium–plant interface is transduced by a DMI gene–dependent signaling pathway to activate PPA assembly and associated transnuclear migration (Figure 3). However, the possibility also exists that the locally generated signal could be of plant origin, resulting from contact between the appressorium and the plant cell wall.

In the case of Nod factor signal transduction in root hairs, intracellular Ca²⁺ oscillations are one of the early characteristic cellular responses (Ehrhardt et al., 1996), and it has been proposed that the role of the downstream DMI3 calcium- and calmodulin-dependent kinase is to interpret this Ca²⁺ signature (reviewed in Oldroyd and Downie, 2004). Because dmi3-1 is defective in PPA formation, intracellular Ca²⁺ signaling could also be an essential component of the PPA activation pathway (Figure 3). If this turns out to be the case, then it will be important to establish whether the AM-elicited Ca²⁺ signature is identical or not to the Nod factor–elicited intracellular oscillation pattern.

In our scheme, we have positioned the activation of the M. truncatula ENOD11 gene upstream of PPA assembly, because we have observed expression of the P<sub>ENOD11</sub>:GFP-HDEL reporter in epidermal cells contacted by appressoria but without visible PPAs (Figure 2G). Previous experiments have shown that the P<sub>ENOD11</sub>:GUS reporter is not transcribed in a dmi2 mutant background despite the formation of surface appressoria (Chabaud et al., 2002). Although we have not yet confirmed these findings for P<sub>ENOD11</sub>:GFP-HDEL reporter expression in dmi roots, this result suggests that the DMI genes are required for activating ENOD11 in appressoria-contacted cells. Our present observations also indicate that not all epidermal cells with visible ENOD11 activation below fungal appressoria subsequently develop PPAs, suggesting that other factors are involved in determining which cells are destined to be infected.

Recently, it was shown by Kosuta et al. (2003) that ramifying AM fungi physically separated from the host root are able to produce a diffusible factor that can activate ENOD11 gene expression primarily in cortical tissues and throughout extensive regions of the root. It was further demonstrated that although this plant response is probably specific to AM fungi, it is absolutely not dependent on DMI gene function, because P<sub>ENOD11</sub>:GUS activation by the diffusible factor was unaltered in mutants of all three DMI genes. By contrast, the infection-related ENOD11 expression described here using the P<sub>ENOD11</sub>:GFP-HDEL reporter and by Chabaud et al. (2002) using the P<sub>ENOD11</sub>:GUS reporter is characterized by (1) a requirement for plant–fungal contact (and associated appressorium formation), (2) expression in only a very limited number of cells directly associated with AM infection, and (3) a probable dependence on functional DMI genes. Thus, bearing in mind that the respective signaling pathways leading to ENOD11 transcriptional activation in the two contexts are likely to be quite distinct and that there is no evidence to date that the diffusible factor response actually plays a role during the early steps of the fungal–plant interaction, we consider it extremely unlikely that the factor described by Kosuta et al. (2003) is involved in signaling steps after fungal–plant contact and leading to PPA formation.

Recently, recent studies have revealed that at least two distinct and separable regulatory regions of the ENOD11 promoter are responsible for gene activation during early stages of the Medicago–Sinorhizobium association. The first region is sufficient for upregulating expression in response to Nod factor perception in root hairs, and the second is sufficient for activating gene expression in tissues associated with rhizobial infection (Boisson-Dernier et al., 2005). Furthermore, the ENOD11 promoter region that is required for rhizobial infection-specific expression (including an important AT-rich motif) is also sufficient to drive tissue-specific expression during AM colonization. Although studies now need to be extended to focus on early AM infection stages, these results clearly suggest that certain infection-related gene regulatory mechanisms have been conserved throughout evolution between the two major root endosymbiotic associations.

**Does the PPA Play a Role in Other Endocellular Plant–Microbe Interactions?**

The identification of the PPA and the implication that the host plant actively prepares for AM infection is clearly in agreement with what Parniske (2000) has referred to as the “intracellular accommodation program” for endosymbionts and what Gianinazzi-Pearson and Dénarié (1997) termed the “red carpet” genetic program. This discovery reinforces the analogies that already exist between the Rhizobium and AM endosymbioses (see Introduction), particularly concerning the cellular mechanisms of microbe entry into the host root. In the case of nitrogen-fixing bacteria, the host synthesizes a unique membrane–matrix tube, called the infection thread, which initiates within the curled root hair as a membrane invagination (reviewed in Gage, 2004). Rhizobia enter the infection thread and progress down the root hair by repeated cell division. The infection thread tip grows continuously ahead of the bacteria, following some distance behind the downward-migrating root hair nucleus. In underlying cortical cell layers, transcellular cytoplasmic bridges (also called preinfection threads [van Brussel et al., 1992]) are formed in advance of the progressing infection thread. Although equivalent in vivo studies using transgenic GFP tags of plant cellular markers have not yet been performed for Rhizobium infection, Timmers et al. (1999) have shown that there is a dense network of microtubules connecting the nucleus to the infection thread tip in both the root hair and the cytoplasmic bridges. Therefore, we can ask whether a cytoskeletal assembly analogous to the A Mel-elicited PPA is also responsible for infection thread synthesis. Finally, although we did not observe this in our experiments with M. truncatula, it should be mentioned in this context that AM infection through root hairs has been reported for certain monocots and dicots, including pea (Pisum sativum) (reviewed in Guinei and Hirsch, 2000).

There is currently no evidence that the equivalent to preinfection thread–containing cortical cells are formed before or during AM root infection; indeed, we have never observed this very characteristic cellular differentiation in cortical cells underlying epidermal AM infection sites. However, it should be borne in mind that in the case of Rhizobium infection, the bacteria are delivered to the developing nodule primordium in the inner cortex.
(at least in the case of temperate legumes forming indeterminate nodules, such as *Medicago*). On the other hand, AM fungal development within the root cortex is quite different, with a progressive and continual colonization of the inner cortex associated with combined intercellular hyphal growth and penetration of certain host cells to differentiate arbuscules. In this respect, it is interesting that Blancaflor et al. (2001) observed a reorganization of the microtubule cytoskeleton in noncolonized cortical cells adjacent to arbuscule-containing cells, suggesting that some kind of signaling between the AM fungus and plant cortical cells occurs before penetration (Harrison, 2005). Thus, the in vivo cellular studies presented in this article should now be pursued to follow AM colonization of outer and inner cortical tissues once the epidermal barrier has been traversed.

Finally, it is important to ask whether analogies can be made with plant infection by certain hemibiotrophic fungi. For example, in the case of a number of *Colletotrichum* species, there is an initial biotrophic phase during which penetration hyphae differentiate specialized structures in epidermal cells that are surrounded by an invaginated plant membrane and interface matrix (reviewed in Mendgen and Hahn, 2002). Although it is not yet understood at the cellular level how the plant host elaborates the membrane invagination and matrix, it is conceivable that this process may be mechanistically related to endosymbiotic infection. Therefore, a comparison of the infection strategies of symbiotic and pathogenic fungal biotrophs and the contributions of the host plant is a major challenge for future research.

### METHODS

#### Plant and Fungal Materials

Experiments were performed with *Agrobacterium rhizogenes*–transformed root cultures derived from both wild-type *Medicago truncatula* Jemalong A17 and two mutants, *dmi2*-2 (TR26; Sagan et al., 1995) and *dmi3*-1 (TRV23; Sagan et al., 1998), kindly provided by G. Duc (Institut National de la Recherche Agronomique). The AM fungal partners used in this study are *Gigaspora gigantea* and *Gigaspora rosea*. Spores of *G. gigantea* were produced using clover (*Trifolium repens*) culture on sand, surface-sterilized, and stored at 4°C according to Bécard and Fortin (1986). Spores of *G. rosea* (DAOM 194767) were produced in vitro using *M. truncatula* hairy roots instead of *Daucus carota* hairy roots. *G. gigantea* has the advantage of possessing a yellowish cytoplasmic autofluorescence (Sejalon-Delmas et al., 1998), greatly facilitating the coordinate staining of roots with the lipophilic probe FM 4-64 (Molecular Probes) to identify putative plant plasma membrane structures was performed by Haseloff et al. [1997] and kindly provided by Jim Haseloff, Cambridge University, UK. In all three cases, the constructs are driven by the constitutive cauliflower mosaic virus 35S promoter and cloned between the T-DNA borders of plant binary vectors. To make the *P_{ENDOD1}:GFP-HDEL* construction, the 35S promoter from *mgfp4-ER* was excised by digestion with HindIII and BamHI and replaced by the entire 2.3-kb *M. truncatula* ENDOD11 promoter (Journet et al., 2001). The four vectors were individually transformed into the *A. rhizogenes* strain Arqua-1, which was then used to introduce the respective constructs into roots of *M. truncatula* Jemalong A17 and the appropriate *dmi* mutants using the transformation protocol described by Boisson-Dernier et al. (2001). In this procedure, which avoids laborious plant regeneration, cotransformed roots expressing both the Ri T-DNA and the binary vector T-DNA are generated after *A. rhizogenes* inoculation of sectioned seedling radicles. A number of cotransformed roots for each marker were selected based on root GFP fluorescence levels, excised from the so-called composite plant, and then propagated in vitro on M medium (Boisson-Dernier et al., 2001) to establish cloned lines and at the same time eliminate contaminating *A. rhizogenes*. Identical patterns of fluorescence labeling were observed for independent transgenic root clones expressing each of the four markers, and a high-level-expressing clone for each marker was selected for further studies. The pattern and intensity of transgene expression in cloned roots were found to be completely stable throughout vitro root subculturing, and the presence of the markers had no observable effect on root growth or on the interaction with the AM fungus.

**Conditions for in Vivo Microscopic Observation of *M. truncatula* Root Infection by *Gigaspora* AM Fungi**

The targeted AM inoculation technique developed by Chabaud et al. (2002) for studying early stages of the symbiotic association between *Gigaspora* species and *A. rhizogenes*–transformed root cultures of *M. truncatula* was adapted for continuous observation with the confocal microscope. Germinated spores of either *G. gigantea* or *G. rosea* were transferred to vertically oriented Petri dishes containing a growing *M. truncatula* transgenic root explant, in such a way that the upward-growing fungal germination hyphae (negative geotropism) contacted the downward-growing roots, thereby facilitating the identification of potential infection sites. The root and fungus were covered with 1 mL of sterile water, on top of which was laid a thin (25-μm) gas-permeable plastic film (bioFOLIE 25; Sartorius AG). The presence of this cover did not interfere with the growth of either organism and the subsequent establishment of fungal infection and colonization of the roots. Within a few days of coculture, hyphal ramifications in the vicinity of the roots could be observed, and appressoria were formed on the root surface as described previously (Chabaud et al., 2002). Importantly, the refractive index of the film is compatible with the use of long-distance water-immersion objectives, thus allowing continuous prolonged microscopic observation, convenient transfer of the dish between the growth chamber and the microscope stage, and minimizing potential contamination of the coculture. The thin aqueous layer between the film and the root ensures a very low endogenous fluorescence in the plant tissues compared with roots growing in air, thus enhancing the sensitivity of GFP detection. Vital staining of roots with the lipophilic probe FM 4-64 (Molecular Probes) to identify putative plant plasma membrane structures was performed by applying 20 μL of a 20 μg/mL aqueous solution to the region of the root of interest (Bolte et al., 2004).

**Confocal Microscopy**

Initial hyphal contact with the root was monitored using a stereomicroscope. The potential infection points were then observed and followed in detail with a Leica TCS SP2 confocal microscope, using a long-distance ×40 water-immersion objective (HCX Apo 0.60). The Ar laser band of...
488 nm was used to excite both the GFP and the G. gigantea autofluorescence. The two signals were discriminated via specific emission windows: 500 to 525 nm for GFP and 590 to 630 nm for fungal autofluorescence. The latter channel was then false-colored in red to maximize contrast in overlapping images. FM 4-64 was visualized with an excitation light of 488 nm (argon laser) and a 630- to 700-nm emission window. The FM 4-64 fluorescence was false-colored in either green or red according to the image.

Accession Number

The accession number for the M. truncatula ENOD11 gene is AJ297721.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. AM-Induced PPA Formation in the Epidermis of M. truncatula Roots Expressing GFP-HDEL and Colocalization with in Vivo FM 4-64 Staining.

Supplemental Video 1. AM Hyphal Penetration into a Root Epidermal Cell Following the Path of the PPA and Showing Progressive Widening of the Fluorescence-Labeled ER Surrounding the Apoplastic Compartment.

Supplemental Video 2. Animated Model Showing Intracellular Dynamics within the Host Epidermis before and during AM Fungal Infection.

ACKNOWLEDGMENTS

We thank Richard Cyr (Pennsylvania State University), Dorus Gadella (University of Amsterdam), Jim Haseloff (Cambridge University), and Boris Voigt (University of Bonn) for providing various cytoskeletal/ER GFP tags. Our thanks also to Alain Jaumeau for assistance with confocal microscopy, to Annie Dedieu for aid in constructing the PENOD11:GFP-HDEL reporter, to Gerard Duc (Institut National de la Recherche Agronomique, Dijon, France) for the dm12-2 and dm13-1 mutants, and to Fernanda Carvalho-Niebel, Etienne-Pascal Journet, and John Esselborn (Laboratory of Plant–Microbe Interactions) for useful criticisms of the manuscript. A.G. received a Marie Curie Fellowship for a 3-month visit to the Laboratory of Plant–Microbe Interactions. This work was supported by grants from the Italian Ministry of Education, University, and Research to P.B. (Fondo Investimenti Ricerca di Base Project Plant/ Microbe Interactions, Progetti di Rilevanza Nazionale 40% 2003–2005, IPP-Consiglio Nazionale delle Ricerche and Centro di Eccellenza per la Biosensoristica Vegetale e Microbica, University of Torino). The San Paolo Company (Torino, Italy) is acknowledged for partly supporting the acquisition of a Leica confocal microscope at the Laboratory of Advanced Microscopy.

Received June 21, 2005; revised September 25, 2005; accepted October 17, 2005; published November 11, 2005.

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Epidermal Cells before Infection

Medicago truncatula

Arbuscular Mycorrhizal Fungi Elicit a Novel Intracellular Apparatus in Medicago truncatula Root Epidermal Cells before Infection
Andrea Genre, Mireille Chabaud, Ton Timmers, Paola Bonfante and David G. Barker

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Supplemental Data /content/suppl/2005/11/04/tpc.105.035410.DC1.html
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