Colonization of Pea Roots by the Mycorrhizal Fungus *Glomus versiforme* and by *Rhizobium* Bacteria: Immunological Comparison Using Monoclonal Antibodies as Probes for Plant Cell Surface Components

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Monoclonal antibodies raised to plant cell surface components expressed during colonization of pea (*Pisum sativum*) nodules by *Rhizobium* were used to investigate common features of pea roots colonized by the arbuscular mycorrhizal fungus *Glomus versiforme*. An extracellular plant glycoprotein recognized by MAC 265 and abundant in the infection threads in pea nodules was completely absent around the mycorrhizal fungus at all stages of host tissue invasion. Antigenic components of the plant glycocalyx recognized by MAC 268 and other antibodies were similarly found on the perisymbiotic membranes that surround the differentiated bacteroids and the fungal arbuscules during the endosymbiotic stage of the interaction. However, the abundance of soluble antigenic components recognized by monoclonal antibody MAC 266 was found to be greatly increased on the interface created around the mycorrhizal fungus during the formation of the arbuscule. Soluble antigens recognized by MAC 266 monoclonal antibody also accumulate in the peribacteroid compartment of pea nodule cells, but some of the antigenic components recognized in mycorrhizal pea roots by MAC 266 may be specific to the mycorrhizal association.

Additional keywords: arbuscular mycorrhizae, cell wall, interface, symbiosis.

Legume plants are a valuable model system with which to investigate symbiotic plant-microbe interactions because their roots can be colonized by at least two important soil microorganisms, the arbuscular mycorrhizal (AM) fungi and the nitrogen-fixing rhizobia. Analysis of successive stages of the infection process by AM fungi, formerly termed VAM (Morton and Benny 1990), and by rhizobia indicates the presence of several features that are common to both symbioses. One important feature is the occurrence of a plant-microbe interface that constantly separates the plant from the invading microsymbiont (Brewin 1990; Bonfante and Perotto 1992). All metabolites and informational compounds that are exchanged between the partners during the establishment of symbiosis must be transported, or transduced, across this interface region, which therefore plays a primary role in the interaction. Another common feature is the fact that invasion of host cells and tissues by AM fungi (Koide and Schreiner 1992) and rhizobia (Long and Staskawicz 1993) occurs without triggering a strong plant defense response. However, a strong plant defense response is induced in nodules when the integrity of the plant-microbe interface is lost (Werner *et al.* 1985).

Pea plants (*Pisum sativum*) form a nitrogen-fixing symbiotic association with *Rhizobium leguminosarum* bv. *viciae* and establish AM symbiosis with several soil fungi belonging to Glomales (Walker 1992). In each of these symbiotic interactions, two successive stages can be recognized in the infection process. The first corresponds to the spreading of the microbial infection inside the plant root tissue. In pea nodules and mycorrhizal roots, this is achieved through the growth of intercellular or transcellular tubular structures; these invasion structures are the infection threads in nodules and the fungal hyphae in arbuscular mycorrhizae. Cytochemical and immunochemical techniques have shown that infection threads (VandenBosch *et al.* 1989a; Rae *et al.* 1992) and fungal hyphae (Perotto *et al.* 1989; Bonfante *et al.* 1990) are similarly bounded by an interface containing plant cell wall components or by the primary plant cell wall when bacteria and hyphae are found in the intercellular spaces.

The second stage of the infection process culminates with the colonization of host plant cells by endosymbiotic microorganisms. In both nodule (Newcomb 1976) and mycorrhizal (Balestrini *et al.* 1992) symbioses, this process is associated with profound morphological changes in the subcellular organization of the plant cytoplasm. Inside these colonized cells, the microsymbionts proliferate and develop into highly pleomorphic forms, the bacteroids in nodules and the arbuscules in mycorrhizae. During this stage, the microsymbiont is separated from the plant cytoplasm by a thin region of interface bounded by a plant-derived perisymbiotic membrane (Brewin 1990). Both peribacteroid membranes (in nodules) and periarbuscular membranes (in mycorrhizae) become actively involved in the bidirectional exchange of nutrients be-

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tween the symbiotic partners (Smith and Smith 1990; Bonfante and Scannerini 1992). However, very little is known about the composition of the periarbuscular membrane, mainly because of the difficulties in isolating enriched subcellular fractions.

The aims of this study were: 1) to investigate whether a 95-kDa plant glycoprotein, abundant in the lumen of infection threads (VandenBosch *et al.* 1989a) and considered a marker for the first stage of infection by *Rhizobium*, was produced during infection by the AM fungus *Glomus versiforme* and 2) to investigate the molecular composition of the periarbuscular compartment in mycorrhizal roots by using monoclonal antibodies previously characterized for their reactivity with components from the peribacteroid compartment in root nodules (Perotto *et al.* 1991). Some of these antibodies were found to be markers in pea root nodules for the second stage of infection involving endosymbiotic colonization and biotrophic interactions.

RESULTS

Tissue invasion.

Spreading of rhizobia within pea root and nodule tissues is typically achieved through the growth of apoplastic tunnels called infection threads, which contain the bacteria. As shown previously, monoclonal antibody MAC 265 identifies a 95-kDa plant glycoprotein secreted into the lumen of the infection threads (VandenBosch *et al.* 1989a; Rae *et al.* 1992). Similarly, Figure 1 shows a light micrograph of the apical region of a pea nodule after immunogold labeling with MAC 265, followed by silver enhancement. Several infection threads containing the MAC 265 antigen could be visualized either as longitudinal or as transverse sections. In addition, many larger infection droplets could be identified on the same section (Fig. 1). When mycorrhizal roots colonized by the AM fungus *Glomus versiforme* were analyzed for the presence of MAC 265 antigen, no traces of immunostaining were found around the large invading fungal hyphae or around the thinner branches of the arbuscules after immunogold/silver enhancement with MAC 265 (Fig. 2A). As with pea nodules, a weak immunostaining was visible along the cell wall when development with the silver reagent was prolonged. To locate the fungus in the root tissue, a contiguous section was counterstained with basic fuschin (Fig. 2B). A positive reaction after immunolabeling of mycorrhizal roots with MAC 265 monoclonal antibody was found in the intercellular spaces between the epidermal and hypodermal root cells and in other intercellular spaces within the root cortex (Fig. 2A). This pattern of immunostaining was a feature also found in uninfected pea roots and in uninfected regions of mycorrhizal roots (Fig. 2C). Control sections, where no primary antibody was used, were completely devoid of silver precipitate (data not shown).

Development of the endosymbiotic stage.

Monoclonal antibody MAC 268 identifies a component of the glycocalyx associated with the plasma membrane and the peribacteroid membrane of pea nodules (Perotto *et al.* 1991; Fig. 3A). Western blot analysis on membrane and soluble fractions obtained from mycorrhizal pea roots confirmed that the MAC 268 antigen was associated only with the membrane fraction (data not shown). On sections of mycorrhizal roots, labeling with MAC 268 antibody was found on the plasma membrane of infected and uninfected cells and around the arbuscules (Fig. 3B). Similarly, five other monoclonal antibodies that react with components of the peribacteroid and

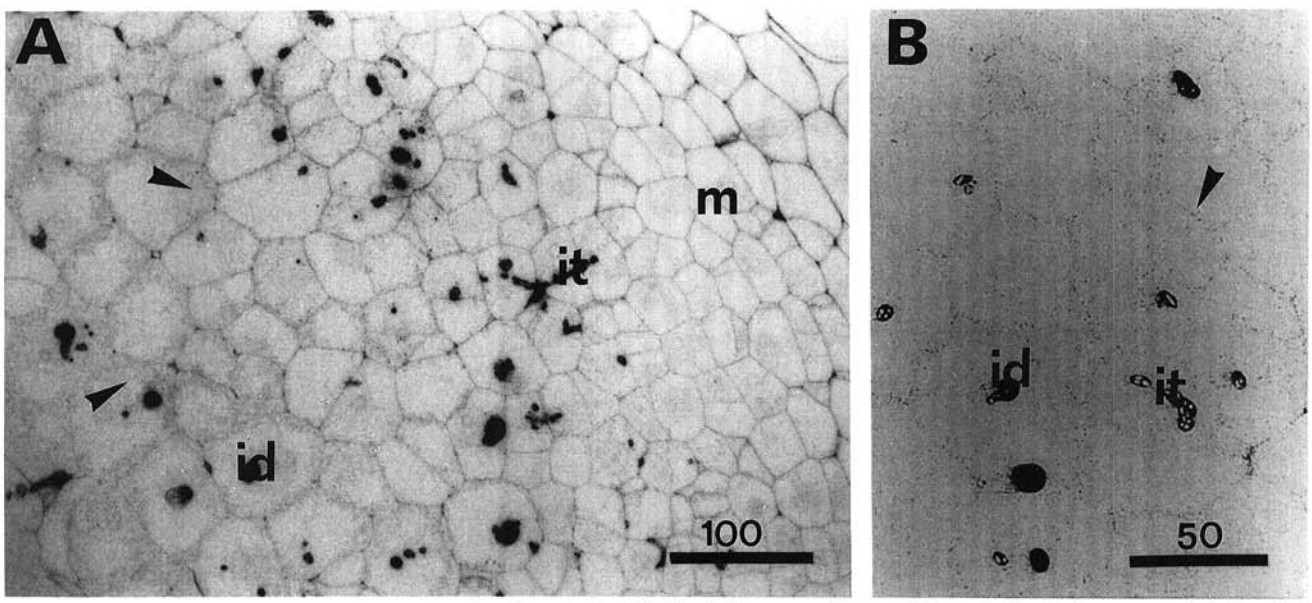

Fig. 1. Expression of the MAC 265 antigen during tissue invasion by rhizobia. **A,** Low magnification of the apical part of a pea nodule in longitudinal section after immunogold labeling with MAC 265 monoclonal antibody and silver enhancement. The section was lightly counterstained with basic fuschin to identify cytological details, and bacteroids are faintly visible (arrowheads) within infected cells. The MAC 265 antigen is visualized as a black deposit on infection threads and droplets, and no immunostaining is detected around the released bacteria. **B,** Higher magnification, showing tightly packed cells from a region near the apical meristem, where no intercellular spaces are found. This section was not counterstained with basic fuschin and shows some MAC 265 immunostaining along the cell wall (arrowhead). Bacteria can be identified as negative images within the matrix material immunolabeled by the MAC 265 antibody. it, infection threads; id, droplets; m, apical meristem. Bar is in micrometers.

plasma membranes (MAC 255, MAC 270, MAC 209, MAC 271, and MAC 272) also reacted positively with periarbuscular membranes.

A most interesting pattern of expression in mycorrhizal roots was observed for the MAC 266 epitope (Fig. 4). This epitope was present only in traces on the surface of plant cells in uninoculated roots (data not shown). Similarly, only traces of immunostaining were found on the interface material surrounding intercellular and transcellular fungal hyphae in the outer cortex of mycorrhizal roots. However, expression of MAC 266 antigen in mycorrhizal roots was suddenly increased in host cells of the inner cortex (Fig. 4) where the fungal hyphae branched out to form the arbuscule (Fig. 5A). In these cells, the MAC 266 epitope was localized not only on the interface formed between plant and fungus, but also at the periphery of the host cell. Immunostaining with MAC 266 on the outer surface of these plant cells was particularly strong in those intercellular regions in direct contact with the fungal hyphae (Fig. 4B). Ultrastructural analysis of infected host cells in the inner root cortex (Fig. 5) confirmed that the MAC 266 antigen was abundant on the apoplastic material laid down by the root cell at the contact point with the fungal hypha (Fig. 5B). In root cells where the fungus formed arbuscules, the MAC 266 antigen was abundant on the thin interface enclosing the arbuscular branches (Fig. 5C) and also around the collapsed arbuscular branches (Fig. 5D) and the residual fungal clumps (Fig. 5E).

Soluble and membrane-associated components of mycorrhizal and nodulated roots were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and analyzed by Western blotting to investigate the mobility of the antigen carrying the MAC 266 epitope. As shown in Figure 6, the pattern of immunostaining with MAC 266 revealed the presence of similar glycoprotein bands in uninoculated, mycorrhizal, and nodulated roots. However, a soluble component of about 50 kDa that was found in the soluble fraction of the mycorrhizal sample was absent from the corresponding fraction from uninoculated or nodulated roots.

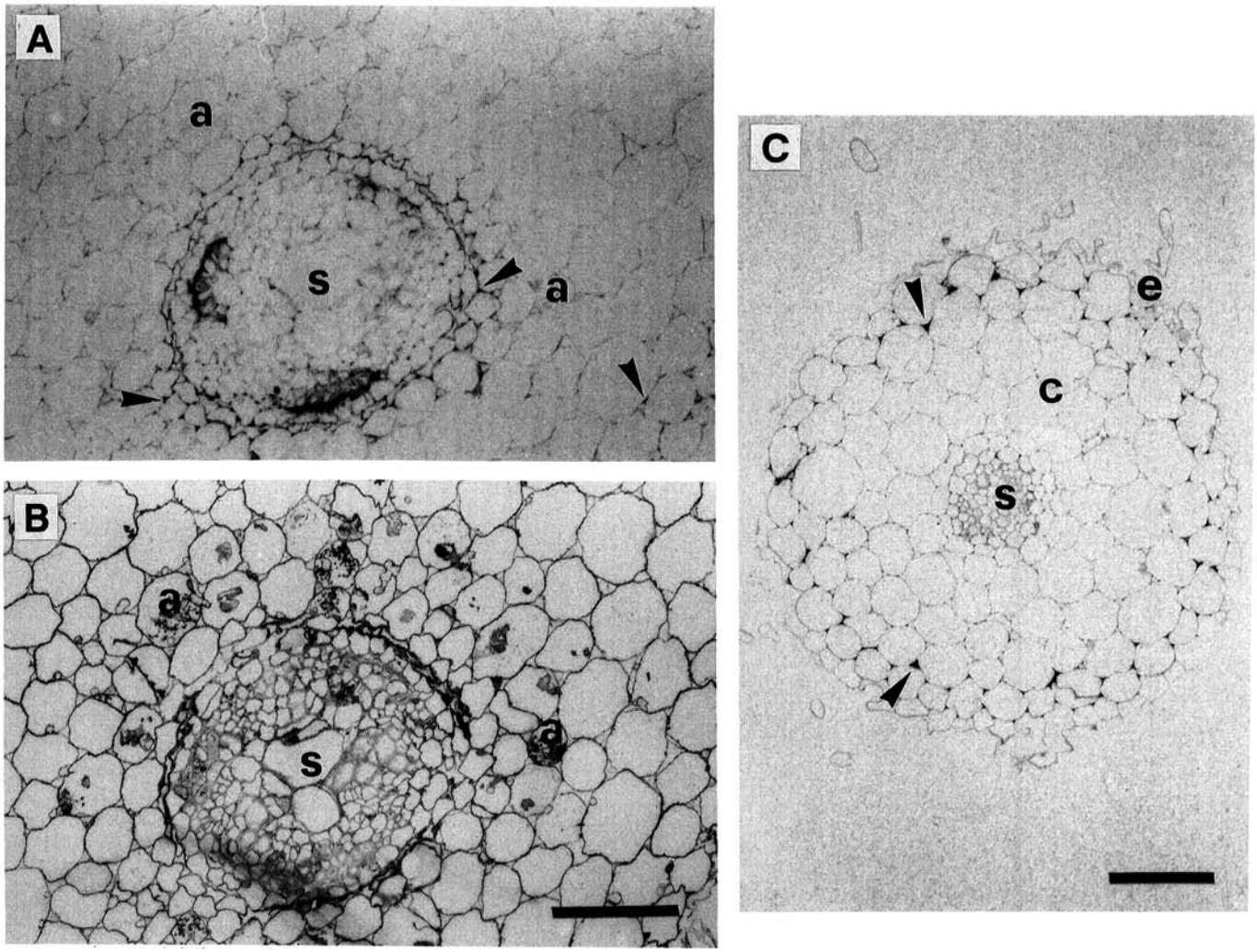

Fig. 2. Expression of the MAC 265 antigen during tissue invasion by arbuscular mycorrhizal fungi. **A,** Transverse section of a pea root colonized by the arbuscular mycorrhizal fungus *Glomus versiforme* and immunogold-labeled with MAC 265 monoclonal antibody followed by silver enhancement. The MAC 265 epitope is not expressed around the fungal hyphae or the arbuscules. Prolonged silver enhancement resulted in a low level of immunostaining on the cell walls and on some cell junctions (arrowheads). **B,** A contiguous section counterstained with basic fuschin to visualize the position of the fungus within the tissue. **C,** Light micrograph of a transverse section from a young uninfected pea root after immunolabeling with MAC 265 antibody. The section was lightly counterstained with basic fuschin. The MAC 265 immunostaining is localized on the cell junctions beneath the root epidermis (arrowheads). a, arbuscule; e, epidermis; c, root cortex; s, central cylinder. Bar equals 200 μm.

DISCUSSION

The occurrence of common antigenic components in nodules and mycorrhizal roots of soybean has been previously reported by Wyss *et al.* (1990), although their location in the plant tissues was not identified. In this article, several components of the plant-microbe interface have been compared during invasion of pea root tissues by AM fungi and *Rhizobium,* using monoclonal antibodies specifically raised to plant cell surface components.

In contrast to rhizobial infection (Fig. 1), invasion of root tissues by mycorrhizal fungi was not associated with the accumulation of the MAC 265 antigen around the fungal hyphae (Fig. 2). It is interesting to note that many of the plant enzymes linked to the defense response (Lamb *et al.* 1989) are not significantly induced in endomycorrhizal roots (Koide and Schreiner 1992). Enzymes such as glucanases, peroxidases, and chitinases have been found during root colonization by rhizobia (Staehelin *et al.* 1992). By contrast, only low levels of endoglucanase (Lambais and Mehdy 1993), chiti-

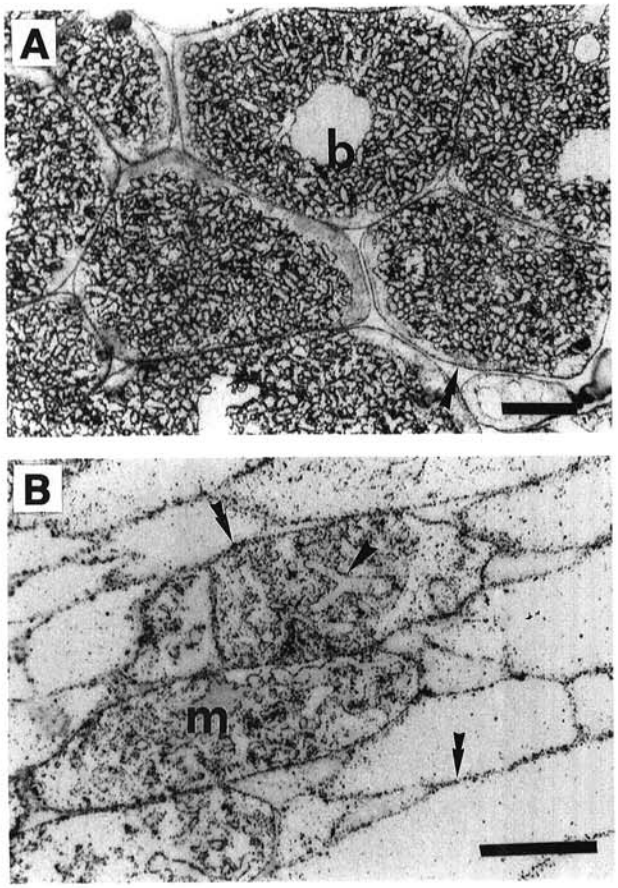

Fig. 3. Expression of epitopes associated with the perisymbiotic membrane. **A,** Light micrograph of infected nodule cells after immunogold labeling/silver enhancement with MAC 268 monoclonal antibody, showing immunostaining on the plasma membrane (double arrowhead) and on the peribacteroid membrane that surrounds the bacteroids. **B,** Cells colonized by arbuscular mycorrhizal fungi, showing a positive reaction after immunolabeling with MAC 268 antibody. Immunostaining is found at the periphery of infected and uninfected cells (double arrowheads) and around arbuscular hyphal branches (arrowhead). b, infected nodule cell; m, arbuscule-containing cell. Bar equals 50 μm.

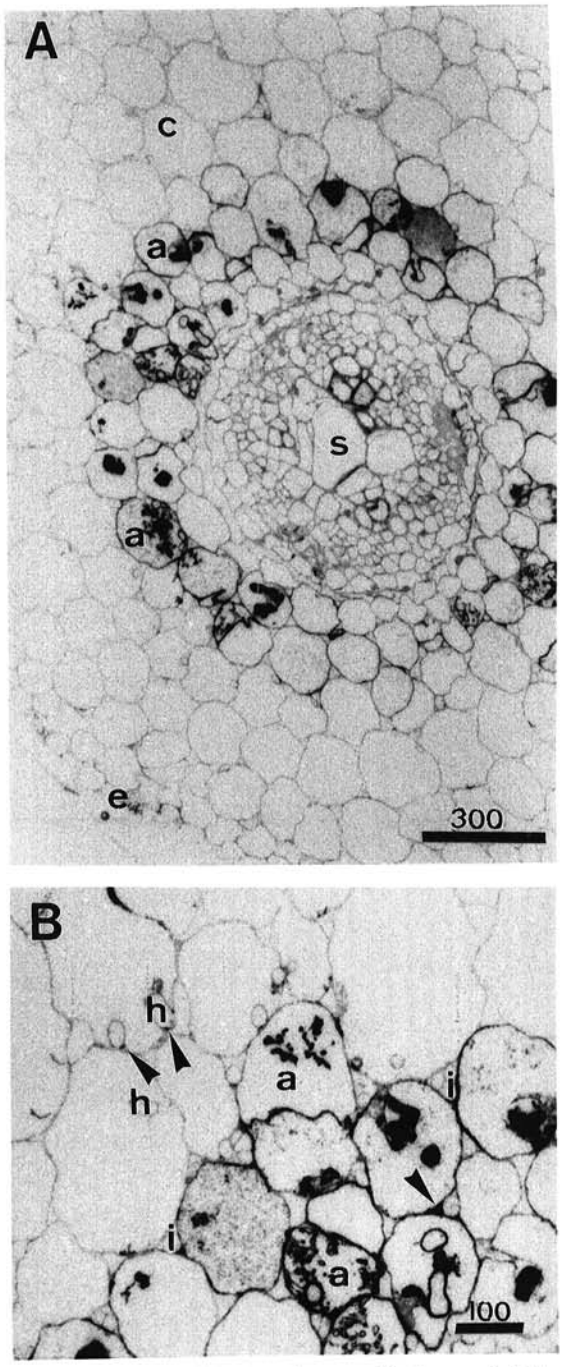

Fig. 4. Expression of MAC 266 antigen in mycorrhizal roots. **A,** Light micrograph showing a transverse section of a mycorrhizal root after immunogold labeling with MAC 266 monoclonal antibody followed by silver enhancement. The section was lightly counterstained with basic fuschin to illustrate the general root cytology. Cells of the inner cortex, near the central cylinder, show strong immunostaining. **B,** Higher magnification of the same root section, showing in more detail the sudden increase of MAC 266 immunostaining in cortical cells harboring arbuscules compared to that in uninfected cortical root tissues and cells containing unbranched fungal hyphae. Small patches of MAC 266 antigen are found where the unbranched hyphae come in contact with the peripheral cell wall (arrowheads). c, root cortex; e, epidermis; s, central cylinder; a, fungal arbuscule; h, unbranched transcellular hypha; i, intercellular hypha. Bar is in micrometers.

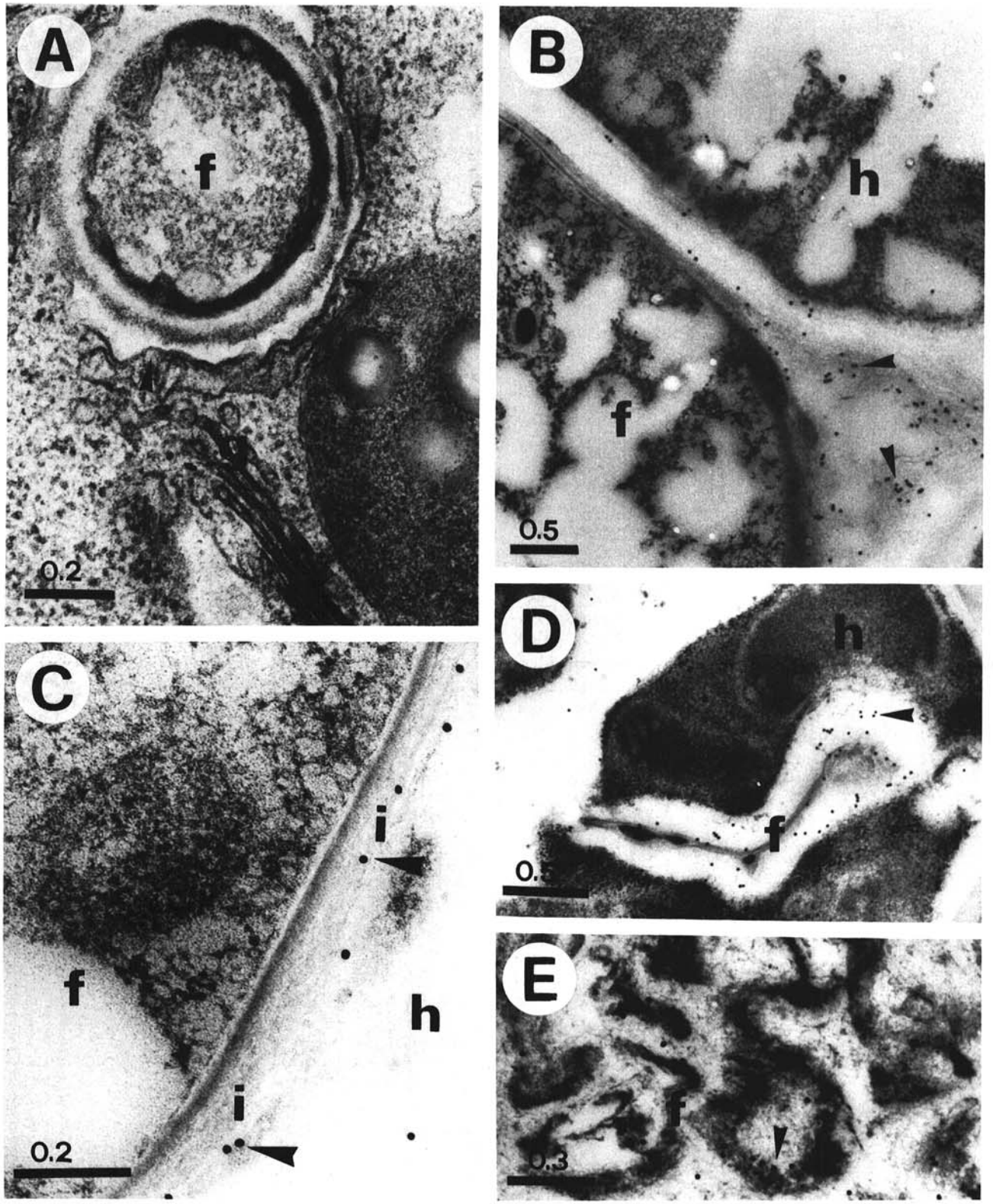

Fig. 5. Electron micrographs showing the subcellular distribution of the MAC 266 antigens in arbuscular mycorrhizal pea roots. **A,** An arbuscular branch surrounded by the plant perisymbiotic membrane (arrowheads) and by interface material. In the plant cell cytoplasm, Golgi bodies are abundant. **B,** Contact point between a host cell of the inner root cortex and a fungal hypha near a penetration point. MAC 266 immunolabeling is distributed on the interfacial wall matrix separating plant and fungus (arrowheads). **C,** The MAC 266 antigen is immunolocalized on the thin interface surrounding the smaller branches of the arbuscule (arrowheads). **D,** Individual arbuscular branches that are collapsing during senescence and are surrounded by an amorphous matrix material containing the MAC 266 antigen (arrowhead). **E,** MAC 266 immunostaining is also present over residual fungal clumps (arrowhead). f, fungus; h, host cell; g, Golgi; i, interface. Bar is in micrometers.

nase (Spanu *et al.* 1989; Lambais and Mehdy 1993), and peroxidase (Spanu and Bonfante 1988) activity have been detected during the early stages of AM fungal infection. Interestingly, the evolutionary origin of AM fungi has recently been dated as 353–462 million years ago, and it has been suggested that these fungi were instrumental in the colonization of land by ancient plants (Simon *et al.* 1993). Thus, the lack of induction of host defense systems by mycorrhizal fungi may be associated with their long history of coevolution, enabling mycorrhizal fungi to colonize up to 90% of land plants. Perhaps another symptom of the "nonaggressive" strategies evolved by AM fungi is the lack of induction of the MAC 265 antigen during the infection process.

Although a clear function for the MAC 265 antigen has not yet been established in nodules, a possible role in plant defense is supported by several observations. In pea nodules, the MAC 265 antigen is overproduced when the tissue is invaded by bacterial mutants altered in their surface lipopolysaccharide; these mutants also induce necrosis and other defense responses in the host plants (Perotto *et al.* 1994). In cultured cells of *Phaseolus vulgaris*, a soluble cell wall glycoprotein recognized by MAC 265 monoclonal antibody was found to be rapidly cross-linked when cells were treated with fungal elicitors (Bradley *et al.* 1992). In the same plant species, a secreted 42-kDa glycoprotein that cross-reacts with the MAC 265 antibody was also found to accumulate in the intercellular spaces of the root cortex (Millar *et al.* 1992). The production and secretion of this 42-kDa glycoprotein in the intercellular spaces increased when the plant was challenged by the fungal pathogen *Colletothrichum lindemuthianum* (Millar *et al.* 1992). A similar defense role in pea may explain the presence of the MAC 265 antigen in the intercellular spaces beneath the epidermis of uninfected roots (Fig. 2C).

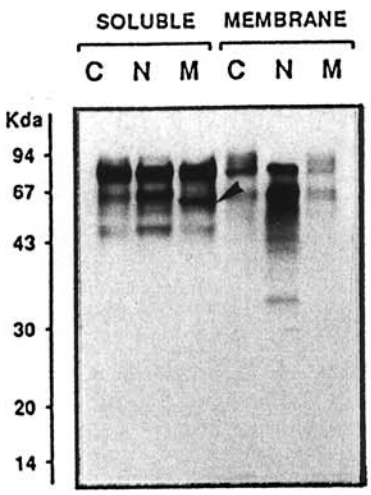

Fig. 6. Size distribution of MAC 266 antigenic components in mycorrhizal and nodulated roots. A Western blot of plant material derived from nodulated and mycorrhizal pea roots was immunostained with MAC 266 monoclonal antibody. Uninoculated (C), nodulated (N), and mycorrhizal (M) pea roots were fractionated in a membrane pellet and a soluble cytoplasmic fraction by ultracentrifugation before separation by sodium dodecyl sulfate-polyacrylamide electrophoresis and electroblotting. Common antigen bands are present in the soluble fraction of the three samples. In addition, an antigen band with mobility unique to the soluble mycorrhizal fraction is indicated (arrowhead).

Our results indicate that some of the molecular components of the plant-microbe interface clearly distinguish between the two main stages of microbial invasion in mycorrhizal as well as in nodule symbiosis. In the first stage, microbial infection is spread through the plant tissues and across plant cells, whereas in the second stage, the microbial partner colonizes individual plant host cells, developing into the differentiated endosymbiotic form. The first stage is characterized by the expression of MAC 265 antigen in infection threads during rhizobial but not mycorrhizal infection. The second stage is associated with the development of a perisymbiotic membrane, recognized by MAC 268 and other antibodies tested in this study. This stage is characterized by the enhanced expression of the MAC 266 antigens around the symbiont, both for the arbuscules of AM fungi (Fig. 4) and for the *Rhizobium* bacteroids (Perotto *et al.* 1991). The clear distinction between these two stages is particularly interesting in relation to arbuscular mycorrhizae, where the interface material is topologically continuous between arbuscules and unbranched hyphae. The function of the MAC 266 antigen is not yet known. However, the gradual accumulation of the MAC 266 antigen during the maturation of bacteroids in pea nodules (Perotto *et al.* 1991) and its massive presence around the collapsing arbuscules in mycorrhizal roots may suggest some involvement in the events leading to microbial senescence.

The phenotype of some symbiotically defective pea mutants identified by Duc *et al.* (1989) suggests that common genetic determinants control the infection process by rhizobia and AM fungi. About half of the nonnodulating (Nod^-) mutant lines that were isolated were also unable to form mycorrhizae (Myc^-). Another group of pea mutants formed only ineffective symbiosis with rhizobia (Nod^+ /Fix^-), and when these mutant plants were tested for AM fungal infection, only large and unbranched fungal hyphae were found, but no arbuscules (Gianinazzi-Pearson *et al.* 1991). These observations support the hypothesis that the two successive stages of the infection process analyzed in this study are controlled by different mechanisms and different host genes. The morphogenetic factors that induce the complex event of arbuscule formation in specific root target cells remain unknown. Components of the host-microbe interface may possibly play a role in this process, and thus study of these components may provide some clues to understanding host-endophyte compatibility in symbiosis.

MATERIALS AND METHODS

Nodulated plants.

Surface-sterilized pea seeds (*P. sativum* 'Wisconsin') were germinated in 200-ml flasks containing nitrogen-free agar medium. One week after germination, they were inoculated with a bacterial suspension of *R. l.* bv. *viciae* strain 3841 and grown as described by Bradley *et al.* (1988). Nodules or nodulated roots were harvested 3 wk after inoculation.

Mycorrhizal plants.

Surface-sterilized pea seeds (*P. sativum* 'Wisconsin') were germinated on filter paper. Three-day-old seedlings were transferred to polyvinyl chloride cylinders filled with sterile quartz sand, and spore clusters of *G. versiforme* (Karst.) Berch were suspended in water and inoculated underneath the

Table 1. Monoclonal antibodies used, with their binding characteristics and localization in pea nodules

Antibody	Epitope of Antigen	Antigen location	Reference
MAC 265	Glycoprotein (95 kDA)	Lumen of infection thread and intercellular space matrix	VandenBosch *et al.* 1989a, Rae *et al.* 1991
MAC 268[a]	Carbohydrate epitope on membrane glycolipids and glycoproteins	Plasma membrane and perisymbiotic membranes	Perotto *et al.* 1991
MAC 266	Carbohydrate epitope on membrane and soluble glycoproteins	Golgi, perisymbiotic membrane, and interfacial material	Perotto *et al.* 1991

[a] MAC 268 was taken as representative of a group of monoclonal antibodies recognizing plant glycocalyx antigens present on the peribacteroid membrane and plasma membranes of pea. Other antibodies tested in this study were MAC 255, MAC 270, MAC 209, MAC 271, MAC 272 (Perotto *et al.* 1991). In all cases, the immunostaining patterns obtained with arbuscular mycorrhizal pea roots were indistinguishable from those obtained with MAC 268.

seedlings. Plants were grown as described by Spanu *et al.* (1989) for 4 wk and were watered regularly with a low-phosphorus-content (3.2 μM) Long Ashton solution.

Fractionation of plant material.

Mycorrhizal pea roots were ground in liquid nitrogen with a pestle and mortar and thawed in homogenization buffer (50 mM Tris-HCl, 10 mM dithiothreitol containing 0.5 M sucrose, 5% insoluble polyvinyl polypyrrolidone, and 5 mM *p*-aminobenzamidine). The sample homogenate was filtered through cheesecloth and centrifuged at 13,000 *g* to separate cell wall debris. After this low-speed centrifugation, the supernatant was collected and ultracentrifuged at 100,000 *g* in a Beckman ultracentrifuge. The membrane pellet and the supernatant containing soluble cell components were collected and mixed with Laemmli sample buffer for separation on 12% SDS-polyacrylamide gels (Laemmli 1970). A comparable weight of nodulated pea roots was fractionated and separated with the same protocol. After separation on SDS-polyacrylamide gel, the molecular components were electrophoretically transferred onto nitrocellulose membranes (Bittner *et al.* 1980) and immunolabeled with antibodies following the procedure described by Bradley *et al.* (1988). No immunostaining was observed on immunoblots where the primary antibody was omitted. Spores of *G. versiforme* were crushed directly in Laemmli sample buffer and analyzed by Western blot. No cross-reactivity on the fungal preparation was found with any of the antibodies tested.

Microscopy and immunolabeling.

Tissue samples from pea nodules and mycorrhizal roots were fixed and embedded for immunocytochemistry as described in VandenBosch *et al.* (1989b). Alternatively, mycorrhizal samples were postfixed with 1% osmium tetroxide and embedded in LR White resin (Polysciences Inc., Warrington, PA), as described by Moore *et al.* (1991). Immunogold labeling was performed on thin section as described by Bonfante *et al.* (1990), followed by a general visualization of polysaccharides with the PATAg reagents (Roland 1978). Immunogold labeling of semithin sections was followed by silver enhancement, according to VandenBosch *et al.* (1989b). Control experiments for immunostaining of thin and semithick sections where the primary antibody was omitted, or an irrelevant antibody was used, resulted in completely unlabeled sections.

Antibodies.

A list of the monoclonal antibodies used in this study is presented in Table 1. All were obtained from fusion experiments that used spleen cells derived from immunized LOU/C rats.

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