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Immunodetection of Infection Thread Glycoprotein and Arabinogalactan Protein in Wild Type *Pisum sativum* (L.) or an Isogenic Mycorrhiza-Resistant Mutant Interacting with *Glomus mosseae*

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Abstract

Monoclonal antibodies were used to detect infection thread glycoproteins (MAC 204, MAC 236, MAC 265) and arabinogalactan proteins (AGPs) (JIM 8, MAC 207) in roots of nod$^+$ myc$^+$ pea (*Pisum sativum* L.) and an isogenic nod$^-$ myc$^-$ pea mutant, inoculated with *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe. MAC 236 and MAC 207 gave strong responses for extracts of all roots in a dot immunobinding assay (DIBA) and were used for immunogold localization of corresponding antigen in root tissues. MAC 236 antigen accumulated in wall appositions induced by the mycorrhizal fungus in outer root cells of the resistant nod$^-$ myc$^-$ mutant, but not in epidermal cells of nod$^+$ myc$^+$ plants. In the latter, MAC 236 antigen was localized in wall material deposited around hyphae penetrating parenchyma cortical cells and around large branches of intracellular arbuscules, but not in the interfacial material or on the host membrane surrounding more finely branched arbuscule hyphae. The antigen accumulated again around wall remains of senescent hyphae. MAC 207 labelled plant plasma membrane, and antigen was also localized in wall appositions below appressoria in epidermal cells of the nod$^-$ myc$^-$ mutant, together with ß-1,3 glucans detected using a monoclonal antibody. The epidermal cell wall of nod$^+$ myc$^+$ roots was unreactive to MAC 207 but related AGP was associated with

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the interface between the fungal wall and host membrane at all stages of arbuscule development in parenchyma cells of nod$^+$ myc$^+$ plants. The possible role of these different molecular components in cellular interactions between the fungal symbiont and root tissues in the nod$^+$ myc$^+$ and nod$^-$ myc$^-$ plants is discussed.

Keywords : arbuscular mycorrhiza, infection thread glycoprotein, arabinogalactan protein, *Pisum sativum*, mycorrhiza-resistant mutants

1. Introduction

Establishment of arbuscular mycorrhizae (AM) requires many steps from the initial recognition events between the two symbionts up to development of the fungus in the root tissue and arbuscule formation within parenchyma cells. The molecular bases of these processes, or the plant-fungal interactions underlying them, are still unclear. Mycorrhiza-resistant isogenic pea mutants (Duc et al., 1989), which are also altered in their ability to nodulate (nod$^-$ myc$^-$ phenotype), all stop fungal development at the root surface and form wall appositions in epidermal and hypodermal cells in contact with appressoria (Gianinazzi-Pearson et al., 1991; Gollotte, 1994). More detailed analyses of nod$^-$ myc$^-$ mutants have shown that some components of these paramural deposits are characteristic of defence reactions elicited by pathogenic fungi. In particular, they contain phenolic compounds, ß-1,3 glucans (callose) and pathogenesis-related PR-1 protein, leading to the suggestion that complex regulatory interactions between symbiosis and defence genes may exist in arbuscular mycorrhiza development (Gollotte et al., 1993; Gollotte, 1994; Gollotte et al., 1995). In fact, intracellular penetration of the fungus in parenchyma root tissue during mycorrhizal infections can induce some weak or transient host defence-like reactions (Gianinazzi-Pearson, 1994). These include the deposition of wall-like material around penetrating hyphae, the amount of which diminishes in the interface between the developing arbuscule branches and the symbiotic host membrane. Cellulose and pectic components persist in this reduced interfacial material (Bonfante-Fasolo et al., 1990) and defence-associated molecules like hydroxyproline-rich glycoproteins (HRGP) and pathogenesis-related (PR-1) protein have been identified throughout the interfacial zone (Bonfante-Fasolo et al., 1991; Gianinazzi-Pearson et al., 1992). Plant factors involved in the complex events accompanying AM formation have not yet been identified but molecules in the host-microbe interface may be essential to the establishment of morphofunctional compatibility between symbionts (Gianinazzi and Gianinazzi-Pearson, 1992; Bonfante and Perotto, 1992).

Previous studies have also indicated the accumulation of some similar proteins and glycocalyx components during host-microbe interactions in arbuscular mycorrhiza and nodule symbioses (Gianinazzi-Pearson et al., 1990; Wyss et al., 1990; Perotto et al., 1994). This, together with the constant association of a nod$^-$ phenotype with the myc$^-$ character in pea mutants (Duc et al., 1989), could signify that common events may be involved in the establishment of both associations. In order to better understand symbiont interactions in arbuscular mycorrhiza, we have investigated the presence of glycoproteins, some of which are known to accumulate during nodule development in the legume-*Rhizobium* symbiosis, in interfaces formed between nod$^+$ myc$^+$ or nod$^-$ myc$^-$ pea roots and the AM fungus *Glomus mosseae*. Monoclonal antibodies recognizing either extracellular infection thread glycoprotein (Bradley et al., 1988; Vandenbosch et al., 1989) or arabinogalactan glycoprotein (AGP) (Knox et al., 1989; Pennell et al., 1989) were used to detect these components in tissues and localize them at the cellular level.

2. Material and Methods

Plant inoculation

Pea (*Pisum sativum* L.) cv. Frisson and an isogenic mycorrhiza-resistant mutant (nod$^-$ myc$^-$, P2, Duc et al., 1989) were grown between Millipore cellulose nitrate membranes (0.45 mm pore size; 47 mm diameter) on which sporocarps of *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe (BEG 12) had been previously germinated, as described by Giovannetti et al. (1993). Controls were obtained under the same conditions in absence of the fungus and plants were harvested 15 days after inoculation. Nodules do not develop in this experimental system.

Nodules were obtained by inoculation of cv. Frisson pea roots with *Rhizobium leguminosarum* bv. *viciae* strain 1007 (kindly provided by N. Amarger, Laboratoire de Microbiologie des sols, INRA, Dijon) (Sagan et al., 1993).

Protein extraction and immunobinding assays

Uninoculated roots, roots inoculated with *G. mosseae*, or nodules were ground at 4°C in 50 mM Tris buffer, pH 7.5, containing 10 mM dithiothreitol, 0.5 M sucrose and 5% (w/v) insoluble polyvinyl polypyrrolidone (Bradley et al., 1988). Extracts were centrifuged 30 min at 8,000g and the supernatants collected. The presence of antigens in the root extracts was tested by a dot immunobinding assay (DIBA) according to Sanders et al. (1992). 1 ml of each extract was deposited on nitrocellulose sheets which were then saturated with 0.5% tris-

casein buffer, pH 7.6, for 30 min and incubated overnight with the primary antibody (MAC 236, MAC 204, MAC 265, MAC 207, JIM8 and MAC 206; 1:100 dilution). The characteristics of the monoclonal antibodies (kindly provided by N.J. Brewin, J. P. Knox and K. Roberts, John Innes Institute, Norwich, England) are listed in Table 1. Revelation of antibody binding was performed using an alkaline phosphatase-conjugated goat anti-rat antibody (1:1,000 dilution). Western blot analyses were performed using each monoclonal antibody after separation of proteins of root and nodule extracts by SDS-PAGE, as described by VandenBosch et al. (1989). Analyses were repeated in three different experiments.

Table 1. Characteristics of monoclonal antibodies used to study *Pisum sativum-Glomus mosseae* interactions

Antibody	Antigen	Reference
MAC 204 MAC 236 MAC 265	Extracellular and infection thread matrix glycoproteins	Bradley et al. (1988) VandenBosch et al. (1989)
MAC 206	Peribacteroid, periarbuscular and plasma membrane oligosaccharides	Bradley et al. (1988) Gianinazzi-Pearson et al. (1990)
JIM 8 MAC 207	Extracellular and membrane-associated arabinogalactan proteins	Knox et al. (1989) Pennell et al. (1989; 1991)

Preparation of specimens for electron microscopy observations and immunocytochemical labelling

Root pieces with mycelium of *G. mosseae* on their surface were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, dehydrated through an ethanol series and embedded in LRWhite resin (Gianinazzi and Gianinazzi-Pearson, 1992). Ultrathin sections for electron microscopy were treated as follows: 30 min in Tris buffer saline (TBS: 10 mM Tris, 154 mM NaCl), pH 7.4, containing 0.05% Tween 20 and 1% BSA; 16h incubation with the primary antibody diluted in TBS-Tween-BSA (MAC 204, 1:2; MAC 236, 1:2; MAC 265, 1:1; JIM8, 1:1; MAC 207, 1:8000; MAC 206, 1:500). After washing, sections were incubated at room temperature for 1 h with a goat anti-rat gold-conjugated antiserum (15 nm) and poststained with 2% aqueous uranyl acetate. For

comparison, double immunolabelling was performed by mixing the MAC 207 rat antibody (1:8,000) with an anti-ß-1,3 glucan monoclonal mouse antibody (1:1,000) (Meikle et al., 1991). Primary antibody binding was distinguished using gold-labelled goat anti-rat (15 nm) and anti-mouse (5 nm) antisera. For each antibody, immunolabelling was performed at least three separate times on several different sections.

Controls consisted of omitting primary antibody or using the unrelated antibody MAC 206 which detects plasmamembrane oligosaccharide components associated with peribacteroid and periarbuscular membranes (Bradley et al., 1988; Gianinazzi-Pearson et al., 1990) (Table 1). Some sections were also pretreated with pronase or 11% sodium metaperiodate (Bradley et al., 1988). For arabinogalactan protein controls, 100 mM L-arabinose (Sigma) or 10 mg/ml gum arabic (Sigma) was mixed with the primary antibody 5 hours before application to sections (Pennell et al., 1989; 1991). Preparations were observed in a Hitachi electron microscope at 75 kV.

3. Results

Detection of infection thread matrix glycoprotein

MAC 204, MAC 236 and MAC 265 antigens belong to a 95 kda glycoprotein family present in pea nodules (Table 1). MAC 236 is specific for an acidic form, whereas MAC 265 detects a neutral form and MAC 204 recognizes both (VandenBosch et al., 1989). Pea root extracts gave positive reactions in DIBA with MAC 236, MAC 265 and MAC 204 but weaker than in nodule extracts (Fig. 1). A 95 kda peptide was specifically detected in nodule extracts by Western blot analyses with MAC 265 and MAC 236, as previously reported by VandenBosch et al. (1989), but no new band was detected in root extracts of mycorrhizal cv. Frisson or the inoculated nod^- myc^- mutants (results not shown). There was no modification in the localization of MAC 265 antigen in mycorrhizal roots, as already reported by Perotto et al. (1994). Since MAC 236 is specific for the other glycoprotein recognized by MAC 204, MAC 236 was chosen for detailed tissue and cellular localization. Plant wall underlying appressoria in cv. Frisson roots always showed only very light, insignificant labelling by MAC 236, in a similar manner to the primary wall not in contact with the fungus (Figs. 2–4). In contrast, accumulation of MAC 236 antigen clearly occurred in the host wall appositions of the nod^- myc^- mutant induced beneath appressoria of the mycorrhizal fungus (Fig. 5). Control treatments without primary antibody (not shown) or with the unrelated rat monoclonal antibody MAC 206 (Fig. 6)

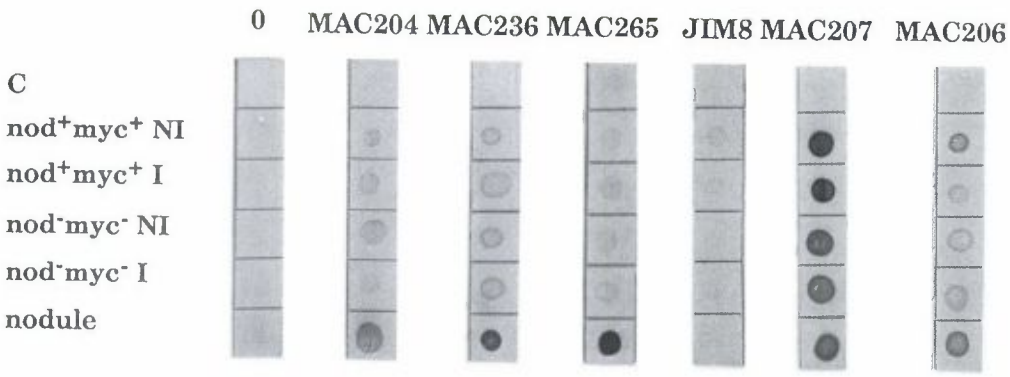


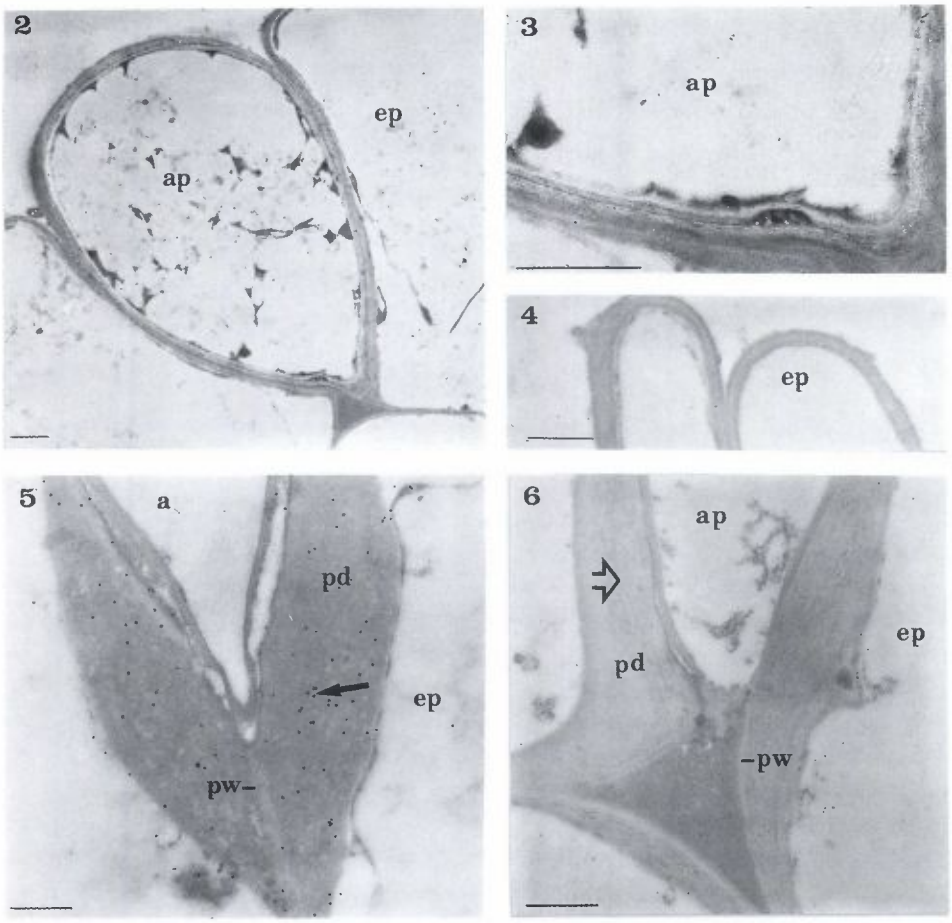
Figure 1. Dot blot experiments. Detection of extracellular glycoproteins in root extracts of uninoculated (NI) or *G. mosseae*-inoculated (I) cv. Frisson (nod^+ myc^+) or mutant (nod^- myc^-) pea plants, and in nodule extracts. Controls were performed by applying the extraction buffer alone (C) or by omitting primary antibody (0). AGPs were revealed by the JIM 8 and MAC 207 antibodies, infection thread matrix glycoproteins by MAC 204, MAC 236 and MAC 265, and glycoproteins usually associated with the perisymbiotic membrane of nodules by MAC 206. In all experiments antibody dilution was 1:100.

gave no labelling on the paramural deposits in the mutant. Furthermore, labelling was inhibited by pretreatment of sections with pronase but was unaffected by sodium metaperiodate oxidation before application of the primary antibody. These observations confirm the specificity of MAC 236 for a protein component (Bradley et al., 1988).

G. mosseae colonized root tissues of cv. Frisson pea further to form a normal arbuscular mycorrhizal infection. In infected parenchyma cortical cells, MAC 236 antigen was only detected in the wall material around penetrating hyphae (Fig. 7) and large, primary arbuscule branches (Fig. 8). No gold granules were observed in the interfacial material or on the periarbuscular plant membrane surrounding thinner arbuscule branches (Fig. 8) but they occurred in the material present at the junction between cells (Fig. 9), as in non mycorrhizal roots, and around clumps of wall remains of senescent arbuscules (Fig. 10). MAC 204 gave similar results to MAC 236 in both cv. Frisson and the nod^- myc^- mutant (not shown).

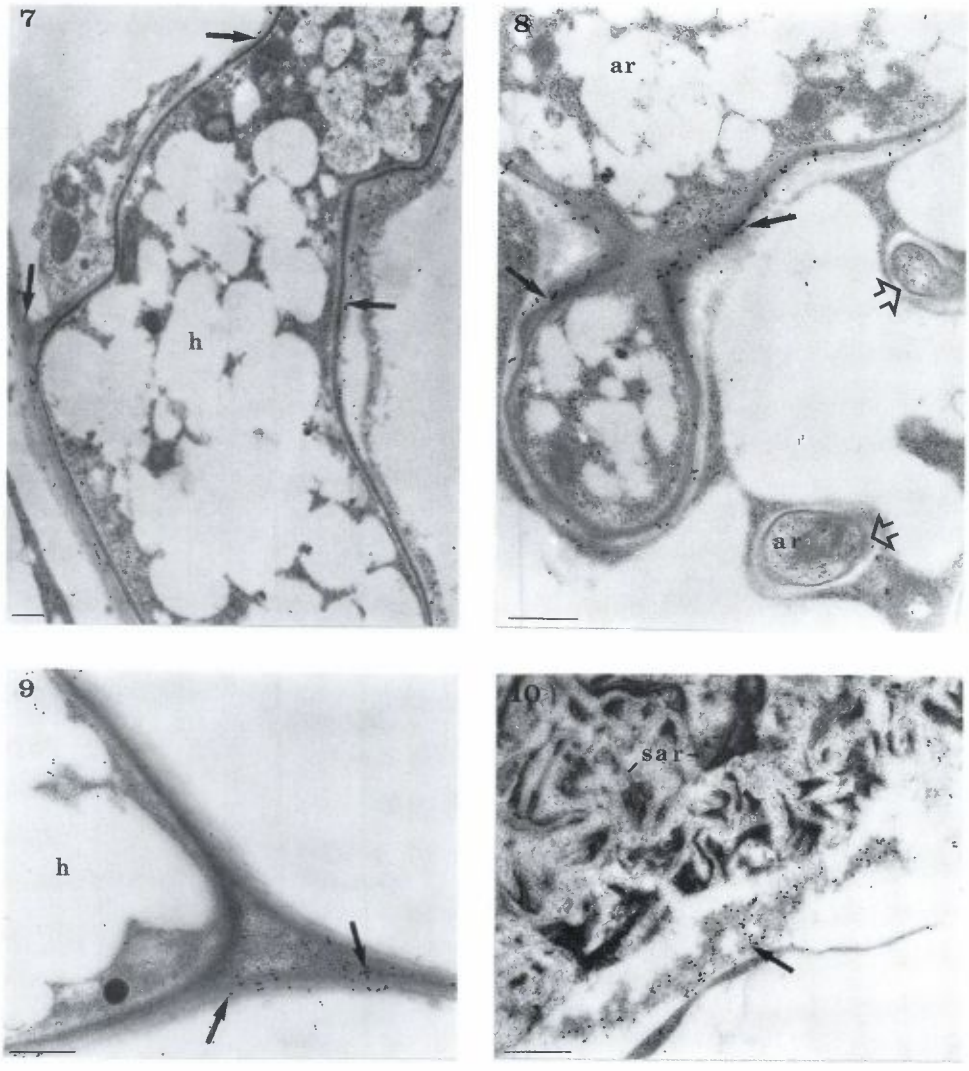
Detection of arabinogalactan protein

JIM 8 and MAC 207 are AGP-specific antibodies (Table 1). Antigen detection in root protein extracts with JIM 8 was weak (Fig. 1) whilst there was a strong



Figures 2–6 Immunolocalization of MAC 236 antigen on the surface of roots of cv. Frisson (nod^+ myc^+) or mutant (nod^- myc^-) pea plants inoculated with *G. mosseae*. Scale bar = 0.5μm. ap, appressorium; ep, epidermal cell; pd, paramural deposit; pw, primary cell wall. **Fig. 2**. An appressorium on a cv. Frisson root. **Fig. 3**. Detail of Fig. 2. No significant labelling can be observed in the plant epidermal wall in contact with the AM fungus. **Fig. 4**. Epidermal cell walls of uninoculated roots with no significant labelling. **Fig. 5**. Numerous gold granules are associated with the paramural deposits in plant cells below appressorium of *G. mosseae* on a mutant root. **Fig. 6**. A control section with the unrelated monoclonal antibody MAC 206 does not show any labelling over the paramural material (open arrow) in a mutant root.

response of all extracts with the MAC 207 antibody in DIBA (Fig. 1). Western blots of extracts from uninoculated roots gave a similar pattern to mycorrhizal or inoculated nod^- myc^- mutant roots (results not shown). MAC 207 antibody was used for immunogold localization of AGPs.



Figures 7–10 Immunolocalization of the MAC 236 antigen in the root of cv. Frisson pea colonized by *G. mosseae*. Scale bar = 0.5µm. h, hypha; ar, arbuscules; sar, senescent arbuscule. **Fig. 7**. Consistent labelling can be observed over the material deposited around a hypha penetrating a cortical cell. **Fig. 8**. Gold granules are present in the interface around large arbuscule branches but are absent from the interfacial matrix around thin arbuscule branch hyphae (open arrows). **Fig. 9**. The MAC 236 antigen is detected in the material present at the junction between cells in intercellular spaces colonized by *G. mosseae*. **Fig. 10**. Material around senescent arbuscules is labelled.

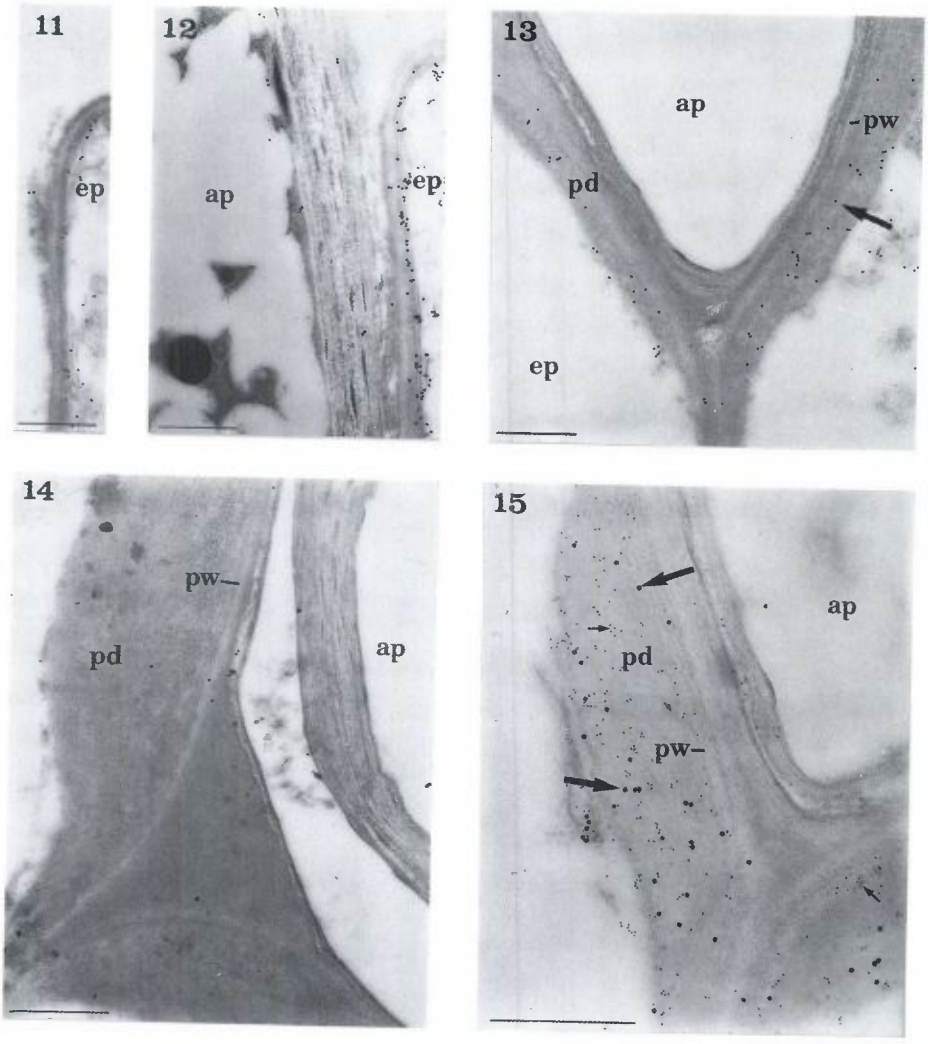
In epidermal cells of cv. Frisson pea roots, MAC 207 antigen was associated with the plant plasma membrane at the inner wall surface. This epitope distribution was not modified in the presence of *G. mosseae* (Figs. 11 and 12). In contrast, antigen accumulated in the paramural deposits formed in epidermal cells of the nod$^-$ myc$^-$ mutant below appressoria of *G. mosseae* (Fig. 13). Immunolabelling was completely eliminated by prior incubation of antibodies with gum arabic (which contains a large quantity of AGPs) (Fig. 14) or arabinose (not shown), confirming the specificity of the MAC 207 antigen detection. Moreover, no gold granules were observed after pretreatment of sections with pronase or metaperiodate, as reported by Pennell et al. (1989). Double labelling of nod$^-$ myc$^-$ mutant roots with MAC 207 and the monoclonal antibody against ß-1,3 glucans clearly demonstrated the presence of both antigens in the plant paramural deposits beneath *G. mosseae* appressoria (Fig. 15).

Occasionally, MAC 207 antigen could also be detected in association with fibrils in the material present at the junction between cells of cv. Frisson pea roots (Fig. 16), whether these were colonized or not by the AM fungus. When mycorrhizal infection developed in roots of cv. Frisson, the antigen was associated with the host membrane and material deposited around penetrating hyphae in parenchyma cortical cells (Fig. 17). It persisted around fine arbuscule hyphae where it was associated with the periarbuscular host membrane, as well as the interfacial matrix (Fig. 18). Disorganized cytoplasm and material accumulating around wall remains of degenerating arbuscules also contained MAC 207 antigen (Fig. 19).

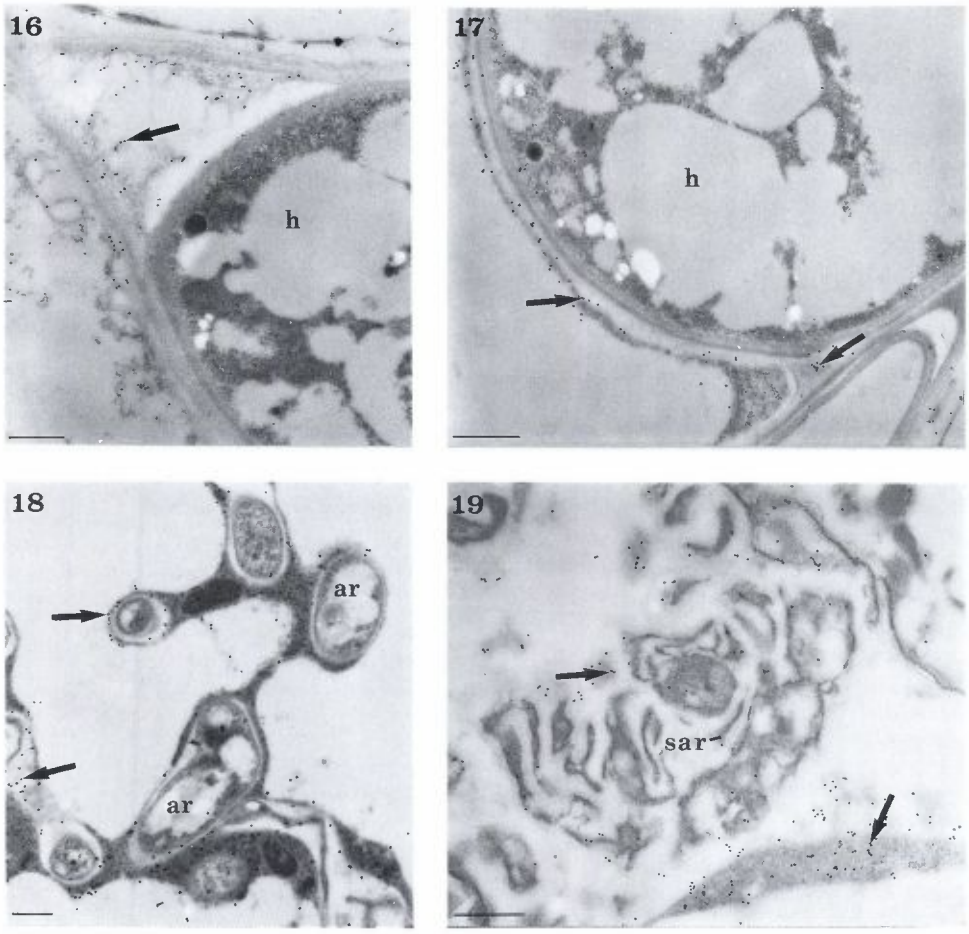
JIM 8 epitope showed no significantly specific localization and antibodies only sparsely labelled some cells in the central cylinder, some junctions between cells and root mucilage (results not shown).

4. Discussion

The results obtained using MAC 204 and MAC 236 are summarized in Table 2. Although MAC 204 and MAC 236 antigens did not increase in epidermal cells of cv. Frisson pea roots in contact with *G. mosseae*, they could be detected in the wall-like material deposited around hyphae penetrating parenchyma cortical cells to form arbuscules. However, they disappeared from the plant-fungal interface with development of the thin arbuscule branches. These results can be compared with those obtained in pea nodules where the MAC 236 antigen was identified within infection thread material produced around bacteria invading roots, but not in the peribacteroid space or membrane of the nodule tissue (VandenBosch et al., 1989). MAC 236 antigen was also clearly associated with



Figures 11–15 Immunolocalization of AGP epitope in epidermal cells of cv. Frisson and mutant (nod⁻ myc⁻) cells using MAC 207. Scale bar = 0.5µm. ap, appressorium; ep, epidermal cell; pd, paramural deposit; pw, primary cell wall. **Fig. 11**. MAC 207 antigen is associated with the plasma membrane in epidermal cells of cv. Frisson. **Fig. 12**. The presence of *G. mosseae* does not induce deposition of the MAC 207 antigen over the plant cell wall in cv. Frisson. **Fig. 13**. In the myc⁻ mutant, MAC 207 antigen accumulates in the paramural deposits induced by *G. mosseae*. **Fig. 14**. Preincubation of MAC 207 with gum arabic inhibits antibody binding to paramural deposits in the mutant. **Fig. 15**. Double labelling of mutant paramural deposits with MAC 207 (15 nm gold granules) (large arrows) and an anti-ß-1,3 glucan monoclonal antibody (5 nm gold granules) (small arrows) shows the simultaneous presence of AGP epitope and the latter.



Figures 16–19 Immunolocalization of MAC 207 antigen (AGP epitope) during *G. mosseae* infection of roots of cv. Frisson. Scale bar = 0.5μm. h, hypha; ar, arbuscule; sar, senescent arbuscule. **Fig. 16.** Gold particles can be observed in the material deposited at the junction between cells in an intercellular space colonized by *G. mosseae*. **Fig. 17**. The membrane and wall material surrounding a hypha penetrating into a parenchyma cortical cell are labelled. **Fig. 18**. The MAC 207 antigen can be detected in the interfacial matrix and on the perisymbiotic membrane around fine arbuscule branch hyphae in a parenchyma cortical cell. **Fig. 19**. Gold particles are present in cytoplasm and material around hyphal remains of a senescent arbuscule.

the paramural deposits formed by the nod$^-$ myc$^-$ pea mutant in epidermal root cells in contact with *Glomus mosseae*. The accumulation of this epitope in such plant wall structures may reflect its relation to the defence response in the mycorrhiza resistant mutant. Interestingly, enhanced MAC 236 glycoprotein

Table 2. Detection of an infection thread glycoprotein and arabinogalactan protein (AGP) in *Pisum sativum-Glomus mosseae* interactions using MAC 236 and MAC 207 monoclonal antibodies, respectively. – or +: absence or presence of immunocytochemical labelling.

Plant cell structure	Antibody	
	MAC 236	MAC 207
Plasma membrane	–	+
Material present at the junction between cells	+	±
***G. mosseae*-inoculated nod⁻ myc⁻ pea mutant:**		
Paramural deposits in epidermal cells at the appressorium level	+	+
***G. mosseae* -infected cv. Frisson pea roots:**		
Epidermal cell wall at the appressorium level	–	–
Host wall around penetrating hyphae in parenchyma cells	+	+
Interfacial matrix around:		
large arbuscule branches	+	+
thin arbuscule branches	–	+
Symbiotic host membrane	–	+
Cytoplasm around senescent arbuscules	+	+

production has been reported to be induced in nodules of legumes subjected to different stress conditions (Iannetta et al., 1993a; b). Disappearance of MAC 236 antigen from the plant-fungal interface with development of arbuscules in cv. Frisson could indicate suppression of a stress-related defence response by the actively developing fungus during the compatible interaction. These observations lend further support to the hypothesis that weak defence responses in roots of myc⁺ plants colonized by a symbiotic fungus may result from control by symbiosis genes over defence gene expression (Gollotte et al., 1993; Gianinazzi-Pearson, 1994; Harrison and Dixon, 1994; Lambais and Mehdy, 1995), such control no longer occurring if a symbiosis-related gene is inactivated as in nod⁻ myc⁻ mutant peas. In contrast, the MAC 265 antigen identified in nodules did not seem to be elicited in arbuscular mycorrhiza confirming previous observations (Perotto et al., 1994). This neutral glycoprotein could therefore be specific to the legume-*Rhizobium* symbiosis.

Although JIM 8 and MAC 207 both detect AGP epitopes, they gave different results with pea roots. The presence of MAC 207-AGP epitope was easily demonstrated by DIBA and Western blotting in nodules, inoculated and uninoculated roots whilst detection of JIM 8 antigen was very weak. These antibodies do, however, recognize two different epitopes in AGPs. JIM 8 is

specific for an antigen only found in certain cells and tissues, whilst the MAC 207 epitope is reported to have a more uniform distribution (Knox et al., 1991; Pennell et al., 1991). Furthermore, the corresponding antigens have been reported to have topographically distinct domains in pollen grains of *Arabidopsis thaliana* where MAC 207 labels the plasma membrane of the vegetative cell and JIM 8 labels the sperm cell wall (Van Aelst and Van Went, 1992).

During the development of a mycorrhizal infection in cv. Frisson pea roots, MAC 207-AGP epitope was particularly associated with the symbiotic arbuscule structures during plant-fungal interactions in the parenchyma tissue. AGPs belong to the hydroxyproline-rich group of glycoproteins associated with plasma membrane and cell wall. Their physiological role is not well established but it is hypothesized that they may be involved in cell to cell recognition, floral histogenesis, differentiation and plant defence reactions (Showalter, 1993). In nodule interactions, AGPs have been reported to be qualitatively different from the AGPs of uninfected roots. They are abundant in the central tissue of the nodule and it has been speculated that they could play a role in cell-to-cell recognition between the symbionts (Cassab, 1986).

MAC 207 epitope also accumulated in the paramural deposits characterizing mycorrhiza resistance in the nod$^-$ myc$^-$ pea mutant. It was localized amongst a ß-1,3 glucan component indicative of the presence of callose, as previously reported by Gollotte et al. (1993). In contrast, ß-1,3 glucans have not been detected in the interfacial matrix around arbuscule branches (Gollotte et al., 1995). Coincidence in the localization of MAC 207 antigen and callose has been shown in pollen tubes of *Nicotiana tabacum* (Li et al., 1992) where AGPs form a network with ß-1,3 glucans. AGPs can precipitate artificial carbohydrate antigen (Yariv's antigen) and act as lectins towards ß-D-glucopyranosyl linkages (Fincher et al., 1983), and it has been proposed that the MAC 207-reactive glycoproteins may bind components of the extracellular matrix to the plasma membrane (Pennell et al., 1989). The simultaneous presence of MAC 207 epitope and ß-1,3 glucans within the resistance-associated material produced in the pea mutant may well reflect elicitation of similar complex molecular interactions by the AM fungus in these genetically altered plants.

The similar pattern of localization of MAC 236 and MAC 207 epitopes in nodules and arbuscular mycorrhizae, together with other previously observed comparable features and the constant presence of the nod$^-$ character in myc$^-$ mutants, lend further support to the hypothesis that common mechanisms may control some early infection events in both symbioses (Duc et al., 1989; Gianinazzi-Pearson et al., 1990; Wyss et al., 1990; Gollotte et al., 1993; Perotto et al., 1994). Some differences do, however exist: for example, MAC 265 epitope which accumulates in nodules is not induced during mycorrhization (Perotto et

al., 1994), and MAC 64 epitope has not been detected in the periarbuscular membrane whereas it occurs in the peribacteroid membrane (Gianinazzi-Pearson et al., 1990). Fossil records indicate that arbuscular mycorrhizae have existed since the Devonian period, shortly after the appearance of the first land plants (Stubblefield and Banks, 1983; Pirozynski and Dalpe, 1989). Legumes appeared later in evolutionary times (between Cretaceous and Oligocene) (Heywood 1971), and their symbiosis with *Rhizobia* is more recent (Young and Johnston, 1989). It is interesting to speculate that part of the plant mechanisms involved in the infection processes leading to nodulation may have evolved from those already established for arbuscular mycorrhizae. In the case of *Rhizobium*-legume symbioses, the development of more complex events of recognition and establishment may have led to the greater specificity between symbionts.

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