Planta (1994) 195:201–209

Planta

Location of a cell-wall hydroxyproline-rich glycoprotein, cellulose and β–1,3-glucans in apical and differentiated regions of maize mycorrhizal roots

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Received: 9 February 1994 / Accepted: 25 April 1994

Abstract. The cell-wall components of the interface compartment in functioning mycorrhizal roots of maize (*Zea mays* L. cv. W64A) have been investigated with the use of immunocytochemistry and enzyme/lectin-gold techniques. The distribution of specific cell-wall probes was determined in the apical and differentiated regions of maize roots in the presence and in the absence of the mycorrhizal fungus, *Glomus versiforme*. Labelling experiments showed that a maize hydroxyproline-rich glycoprotein (HRGP), identified with a specific antibody, was particularly abundant in the apical dividing cells of the root meristem. Cellulose, located with a cellobiohydrolase-gold complex, showed a similar labelling pattern in the walls of both meristematic and differentiated parts of the roots. When the cortex was colonized by the mycorrhizal fungus, the HRGP and cellulose were expressed in two sites: the wall and the interface area created by invagination of the host membrane around the developing fungus. In contrast, in uninfected roots of the same age, they were only present in the inner part of the wall. A specific antibody against β–1,3-glucans demonstrated that these glucans were not laid down at the interface between the plant and fungus, while they appeared to be a skeletal component of the fungal wall, together with chitin.

Key words: Arbuscular mycorrhizae – β–1,3-glucans – Cellulose – Cell wall – Hydroxyproline-rich glycoprotein – *Zea* root meristem

Introduction

Mycorrhizae are tight associations established between the roots of about 90% of land plants and some beneficial soil fungi. During this symbiosis, plants improve their mineral nutrition, while fungi accomplish their life cycle. These events are the result of a balance, mirrored by substantial changes at all levels of metabolic activity, from gene expression to morphogenesis (Bonfante and Perotto 1992).

When a mycorrhizal, and in particular an arbuscular-mycorrhizal fungus colonizes a host tissue, it may either develop between the host cells or actually penetrate them. In the first case, the walls of both partners are in direct contact, while in the second the intracellular hyphae are separated from the host cytoplasm by the invaginated host membrane, producing an interface space of about 50–100 nm between the fungal wall and the host membrane. Affinity techniques have already demonstrated the presence of cellulose, homogalacturonans and hydroxyproline-rich glycoprotein (HRGP) molecules in leek and pea roots at their interface with an arbuscular mycorrhizal fungus, *Glomus versiforme* (Bonfante et al. 1990b, 1991).

The HRGPs are among the best-known protein components of the plant cell wall. In maize, as in many Gramineaceae, cell-wall components (mainly pectins and hemicellulose) differ from those of dicotyledonous species (Carpita and Gibeaut 1993). The protein, cDNA and genomic patterns of maize HRGPs (Kieliszewski and Lamport 1987; Stiefel et al. 1990) show that they are very simple by comparison with dicotyledons. Maize HRGP has been shown to be accumulated in organs rich in dividing cells, in particular in the root meristem, but not in the cortex of differentiated roots, where it is induced during lateral root formation (Stiefel et al. 1990). The gene can also be induced by wounding and ethylene (Ludevid et al. 1990; Tagu et al. 1992).

In laboratory conditions, maize roots establish arbuscular mycorrhizal symbioses (AMs) with typical morphological features (Toth and Miller 1984), even if evident growth effects on host plants have not been reported (Kothari et al. 1990).

Specific antibodies against maize HRGP and β–1,3-glucans and a cellobiohydrolase-gold complex (with affinity to native cellulose) were used in this study: (i) to

Abbreviations: CBH I = cellobiohydrolase; DAPI = 4',6-diamino-2-phenylindole; HRGP = hydroxyproline-rich glycoprotein

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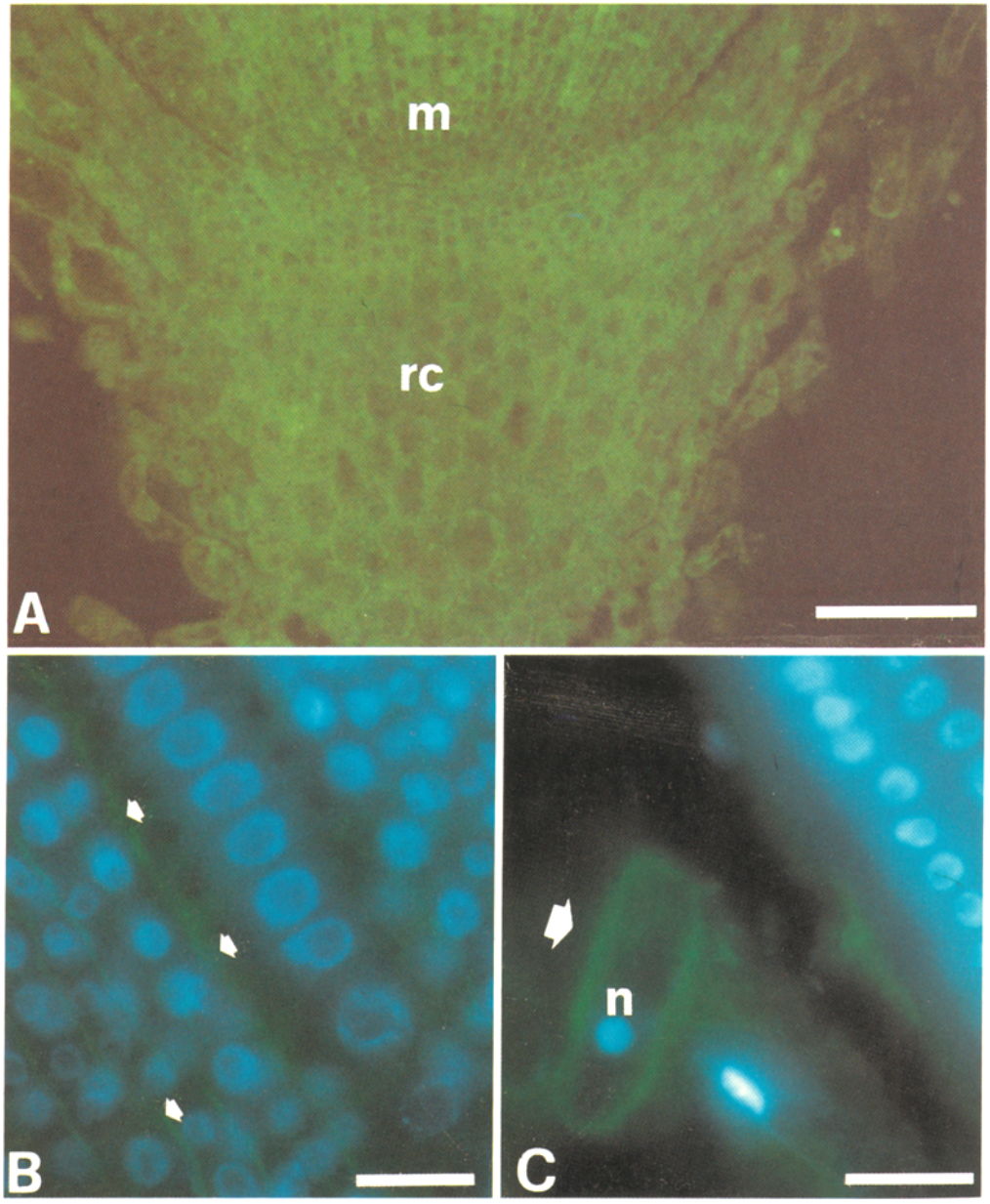

Fig. 1A–C. Immunofluorescence location of HRGP in cryomicrotome sections of maize root tips. **A** Longitudinal median section: all the walls of root cap cells, root meristem and epidermis are fluorescent. ×220; bar = 100 µm. **B** The longitudinal walls of the metaxylem elements (*arrows*) are more fluorescent than the transversal walls. ×640; bar = 25 µm. **C** Strong labelling in a sloughing cap cell. ×700; bar = 25 µm. The nuclei in **B** and **C** are labelled by DAPI-staining. *rc*, root cap; *m*, meristem; *n*, nucleus

locate HRGP in the meristematic and differentiated parts of maize roots in the presence or absence of the mycorrhizal fungus *Glomus versiforme*, (ii) to see whether HRGP molecules surround the intracellular fungus and (iii) to compare the distribution of HRGP with that of cellulose and that of β–1,3-glucans.

Materials and methods

Plant material. Seeds of *Zea mays* L. cv. W64A obtained from plants grown in a greenhouse at CID-CSIC in Barcelona were sown in sterilized quartz sand. The germinated seedlings were watered three times a week with low-phosphorus ($HPO_4^{2-} = 6\ mg \cdot l^{-1}$) Long Ashton solution (Hewitt 1966). Mycorrhizal plants were obtained by inoculating seeds with a spore suspension collected from *Glomus versiforme* (Karst) Berch fruit bodies. Original spores were provided by Dr J. Trappe, Oregon State University, Corvallis, Ore., USA. All plants were maintained in a growth chamber at 24°C with a relative humidity of 75%, and a 16-h day length. Infected and uninfected root samples were collected two months after inoculation with mycorrhizal fungus.

Microscopy and immunocytochemistry. Apical and differentiated segments from uninfected and mycorrhizal roots were fixed in 2.5% glutaraldehyde in 10 mM Na-phosphate buffer (pH 7.2) for 2 h at room temperature. After rinsing with the same buffer, the root segments were postfixed in 1% OsO_4 in H_2O for 1 h, washed three times with H_2O, and dehydrated in an ethanol series (30, 50, 70, 90, 100%; 10 min each step) at room temperature. The root segments were infiltrated in 2:1 (v/v) ethanol/LR White resin (Polysciences Inc., Warrington, Pa., USA) for 1 h, 1:2 (v/v) ethanol/LR White for 2 h, and 100% LR White overnight at 4°C, according to Moore et al. (1991). Semithin sections (1 µm) were stained with 1% toluidine blue for morphological observations.

For the location of HRGPs, a polyclonal antibody against the purified maize HRGP was used raised by injecting the purified proteins into rabbits (Ludevid et al. 1990). For electron microscopy, the antibody was used at dilutions of 1:1000 or 1:2000 on thin

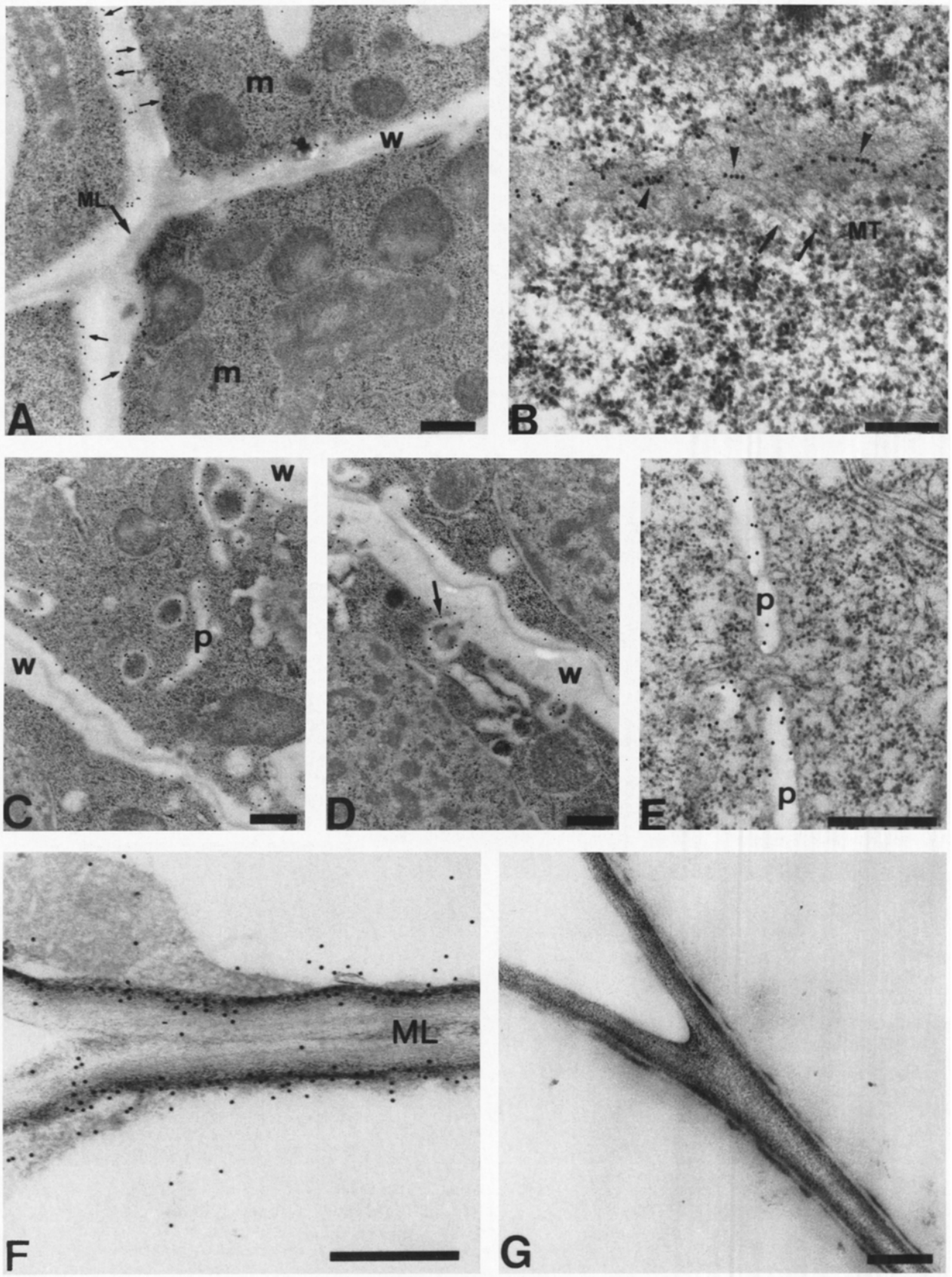

Fig. 2A–G. Immunogold location of HRGP in ultra-thin sections of meristematic cells (**A–E**) and in the differentiated region (**F–G**) of maize root. **A** Gold granules are present at the periphery of the wall (*arrows*). No labelling is found at the junctions among cells. × 19000. **B** Microtubules (*arrows*) are close to the wall which is immunolabelled with the anti-HRGP antibody (*arrowheads*). × 34000. **C–D** Gold granules are present inside large membranous invaginations, which are connected to the cell membrane. × 16000. **E** Magnification of the cell-wall plate with labelling detected inside. × 38000. **F** In the differentiated regions the labelling is found at the peripheral part of the wall, in close contact with the membrane. × 45000. **G** In control experiments, no labelling is present. × 23000. *m*, meristematic cell; *ML*, middle lamella; *MT*, microtubules; *p*, cell-wall plate; *w*, wall. Bars = 0.5 μm

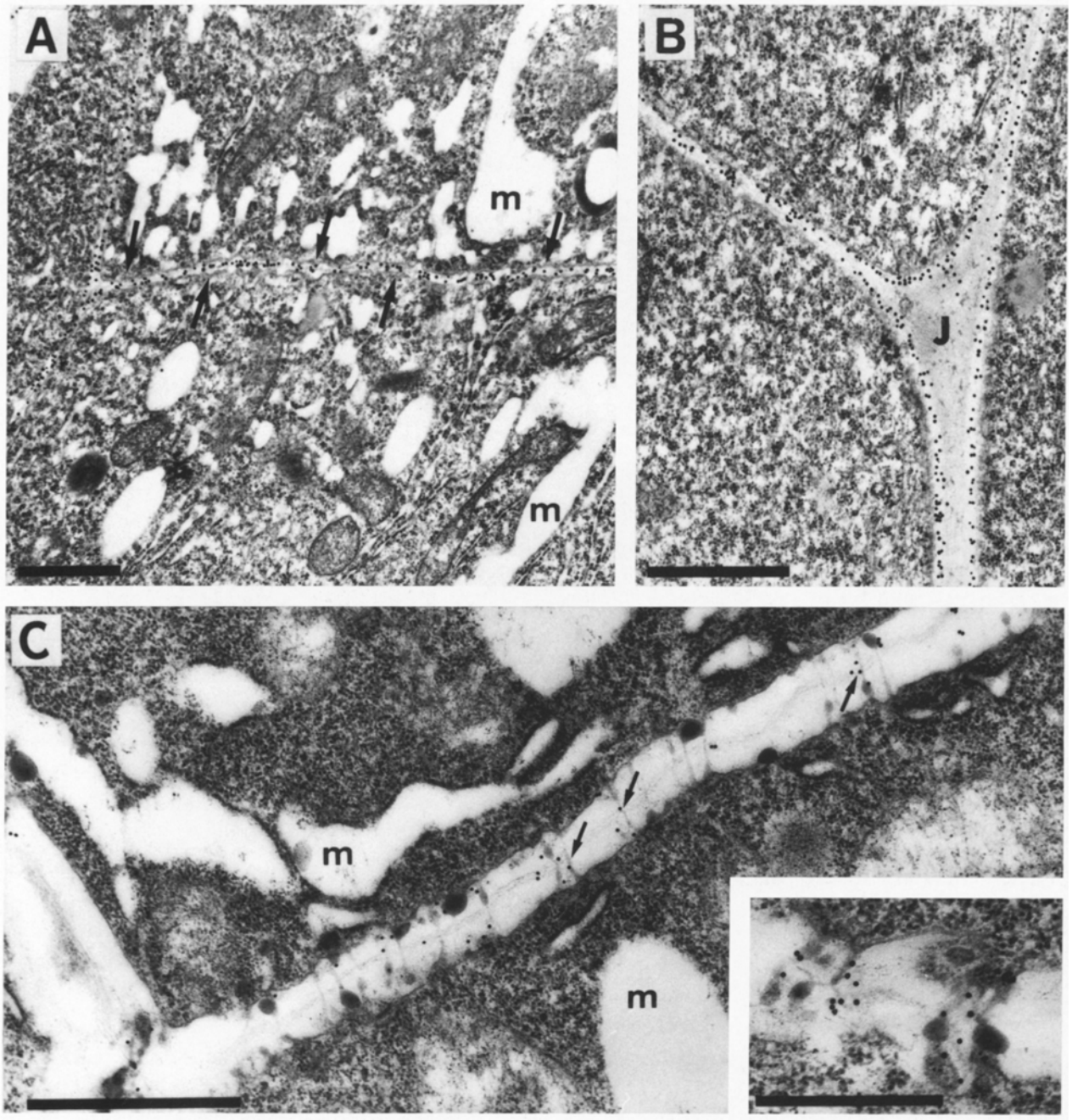

Fig. 3A–C. Localization of cellulose (**A–B**) and β–1,3 glucans (**C**) in ultra-thin sections of maize root tips. **A** Gold granules are present over the thin wall of a dividing cell after CBH-gold complex treatment (*arrows*). × 15500. **B** Labelling is limited to the periphery of the already differentiated wall. No labelling is found at the junction between the cells. × 22000. **C** Labelling is present only on the plasmodesmata (*arrows*) after treatment with an antibody against β–1,3-glucans. × 33000. Bars = 1 μm. *Inset* Detail of the labelling on the plasmodesmata. × 58000. *m*, meristematic cell; *J*, junction among cells. Bar = 0.5 μm

sections. Sections were incubated for 15 min in normal goat serum diluted 1:30 in 0.05 M Tris-HCl buffer with 500 mM NaCl (TBS, pH 7.6) and 0.2% bovine serum albumin (BSA), and treated overnight with the antibody. After washing, sections were incubated with 15-nm colloidal gold-goat antirabbit immunoglobulin complex (Bio Cell, Cardiff, UK) containing 1% BSA (diluted 1:20 in TBS) for 1 h. Labelling specificity was determined by replacing the primary antibody with buffer. Thin sections were poststained with uranyl acetate and lead citrate before observation with a Philips CM 10 transmission electron microscope (Philips, Eindhoven, The Netherlands).

β–1,3-glucans were detected by using a rabbit polyglucopyranose polyclonal antibody (Euromedex, Schiltighem, France; Northcote et al. 1989), following the same protocol used for HRGP; the working dilution was 1:1000. The specifity of the antibody labelling was determined (i) by replacing the primary antibody with buffer and (ii) by preincubating the antibody for 72 h at 4°C with 100 mg·ml^{-1} laminarin (Sigma Chimica, Milan, Italy).

For immunofluorescence, root segments were fixed in 4% *p*-formaldehyde in 50 mM 1,4-piperazinediethanesulfonic acid (Pipes), 5 mM $MgSO_4$, 5 mM EGTA (pH 6.9) for 2 h, washed with 50 mM Pipes (pH 6.9) and left for several days in a solution consisting of 0.5% *p*-formaldehyde and 1.5 M sucrose in Pipes.

Sections (20–30 μm thick) were obtained with a cryomicrotome

(Microm, Heidelberg, Germany), washed with 50 mM PBS (pH 7.2), saturated 1 h with 1% BSA in the same buffer, treated with the primary antibody against HRGP overnight at 4°C, and then washed with PBS. The antibody was used diluted 200–500 times. The anti-rabbit secondary antibody was FITC (fluorescein isothiocyanate)-conjugated and was used at the recommended dilutions (Sigma). Some sections were simultaneously treated with a saturated solution of DAPI (4',6-diamino-2-phenylindole) at 2 $\mu g \cdot ml^{-1}$ to detect nuclear distribution. Sections were then observed with a fluorescence microscope (Universal Zeiss, Göttingen, Germany) equipped with a BP 450–490 excitation filter and a BP 520–560 barrier filter for FITC observations. Alternatively, G 365 and LP 420 filters were used for DAPI.

Controls were performed by omitting the primary antibody step.

Colloidal gold conjugates. β–1,4-glucans and chitin molecules were detected with the enzyme cellobiohydrolase (CBH I, EC 3.2.1.91, kindly provided by Dr B. Vian, Pierre et Marie Curie University, Paris, France) and the lectin wheat-germ agglutinin. Gold complexes and cytochemical labelling were performed as described in Bonfante et al. (1990b) and Vian and Roland (1991).

Results

Location of HRGP and glucans in cell walls of root meristem. When the cryomicrotome sections of root tips were incubated with the antibody, cell walls were uniformly labelled in both the cap and the meristem (Fig. 1A). At higher magnification, the differentiating region showed metaxylem cells which were more fluorescent in their longitudinal than in transversal walls (Fig. 1B). The differentiated sloughing cap cells were also strongly labelled (Fig. 1C). The pattern of labelling was better detectable on the ultrastructural level: in meristematic cells, gold granules were mostly found at the periphery of the wall (Fig. 2A) closely associated with plant membrane invaginations (Fig. 2C,D). Microtubules were sometimes close to the gold granules (Fig. 2B). No labelling was seen in the middle lamella and at the junctions between cells (Fig. 2A,F). Labelling was also present inside the cell-wall plate vesicles forming the developing transversal walls (Fig. 2C,E). Even in the differentiated regions, labelling was limited to the peripheral part of the wall, in close contact with the membrane (Fig. 2F). No labelling was observed when the primary antibody was omitted (Fig. 2G). The labelling pattern was similar when CBH I (a probe for cellulose location) was used: gold granules were spread all over the thin walls of the young and dividing cells, and limited to the periphery of the wall in already differentiated cells (Fig. 3A,B). No labelling was observed when carboxymethylcellulose was added to the gold/enzyme complex (result not shown). The β–1,3-polyglucopyranose antibody only labelled plasmodesmata, which were particularly abundant in the developing transversal walls (Figs. 3C and inset). All the probes led to the same labelling pattern, even when the fungus was present in the differentiated regions (data not shown).

Location of HRGP and glucans in the mycorrhizal interface. The mycorrhizal fungus only colonizes differentiated cells and its presence greatly alters the host cell organization (Bonfante and Perotto 1992). In maize, *Glomus versiforme* produces large intracellular hyphae, which cross cortical cells (Fig. 4A) and often branch to produce a complex ramified structure called an arbuscule. Hyphae of various sizes (from 4–5 to 0.5 μm) colonized the cortical cells, each fungal branch being surrounded by a membrane of host origin (Fig. 4B). Host organelles (nucleus, mitochondria, Golgi bodies) surrounded the hyphae. After the use of the anti-HRGP antibody, in addition to labelling over the wall (Fig. 5A,B), gold granules were constantly deposited in the interface space between the plant membrane and the fungal branches. The la-

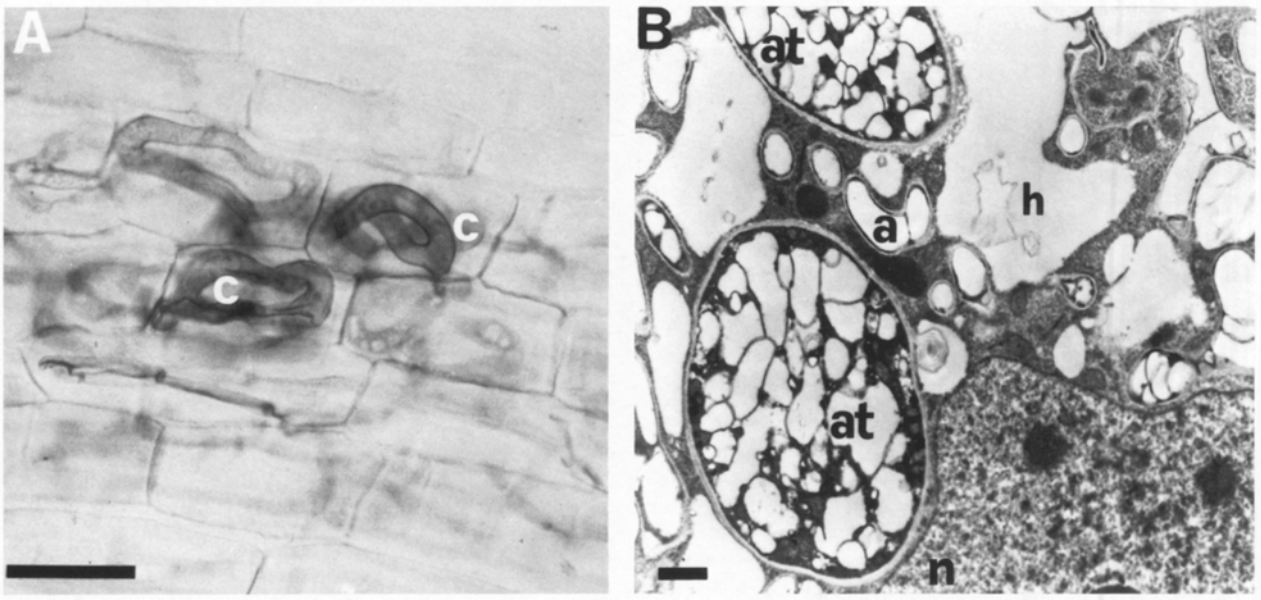

Fig. 4A,B. Mycorrhizal root of *Zea mays*. **A** Cryomicrotome section showing fungal coils (*c*) inside the cortical cells. × 330; bar = 50 μm. **B** Ultrastructure of a mycorrhizal cortical cell. The fungus produces a ramified intracellular structure called an arbuscule, consisting of large (*at*) and thin (*a*) hyphae. × 6000; bar = 1 μm. *n*, host nucleus; *h*, host cell

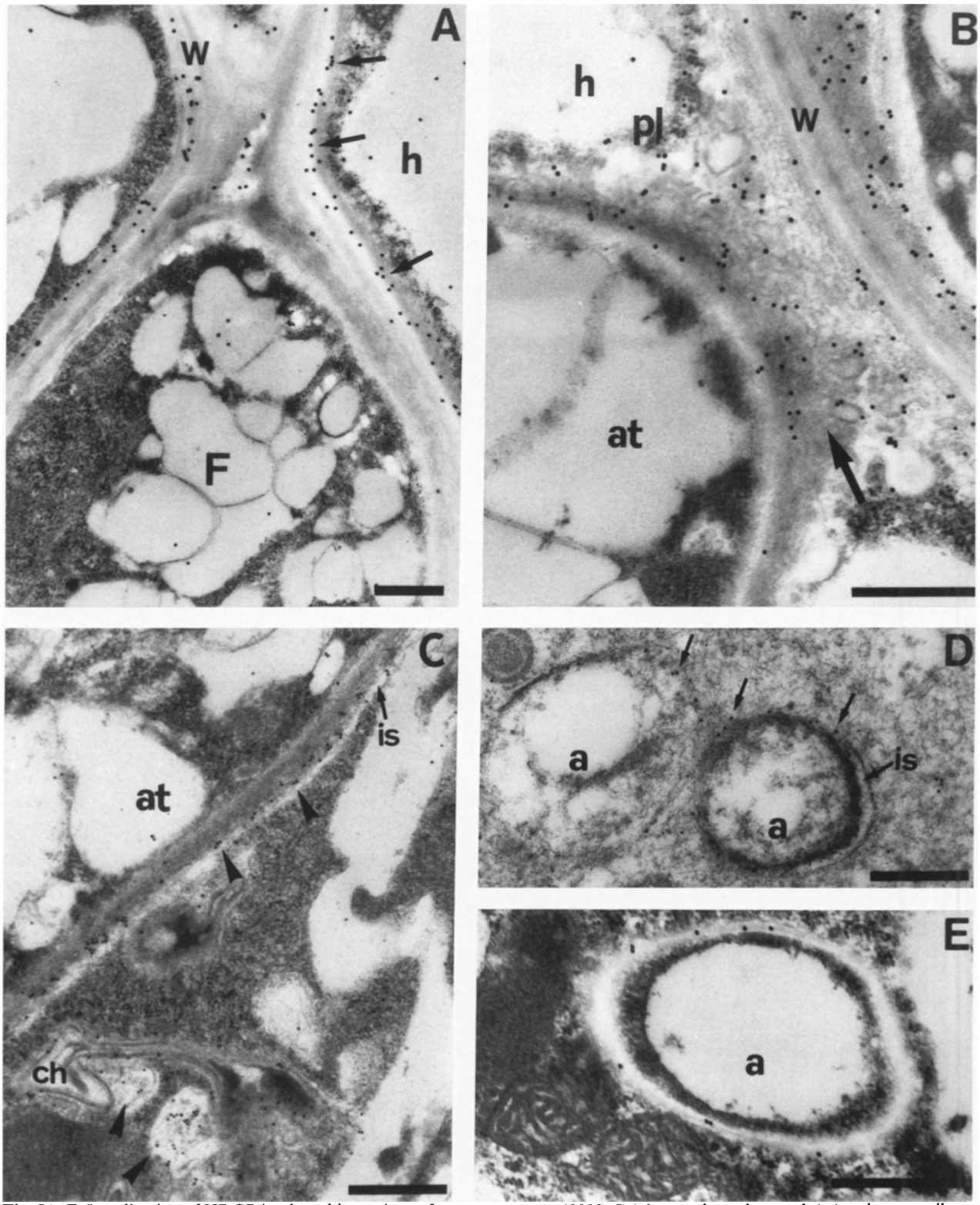

Fig. 5A–E. Localization of HRGP in ultra-thin sections of a mycorrhizal root. **A** The gold granules are regularly present over the host cell wall, particularly in the peripheral region near the plasmamembrane. × 38000. **B** A tangential section of the penetration point shows abundant gold granules (*large arrow*) at the interface zone between the fungus (*at*) and the host. Note the continuity between the perifungal membrane and the host plasmamembrane (*pl*). × 43000. **C** A large arbuscular trunk (*at*) and some collapsed arbuscular branches (*ch*) are shown. Gold granules are present at both types of interface. × 33000. **D–E** The anti-HRGP antibody regularly labels the interface zone around the thin arbuscular branches (*arrows*). **D** × 33000; **E** x45000. *a*, arbuscule thin branches; *at*, arbuscule trunk; *h*, host; *ih*, intercellular hypha; *is*, interface space; *w*, wall. Bars = 0.5 μm

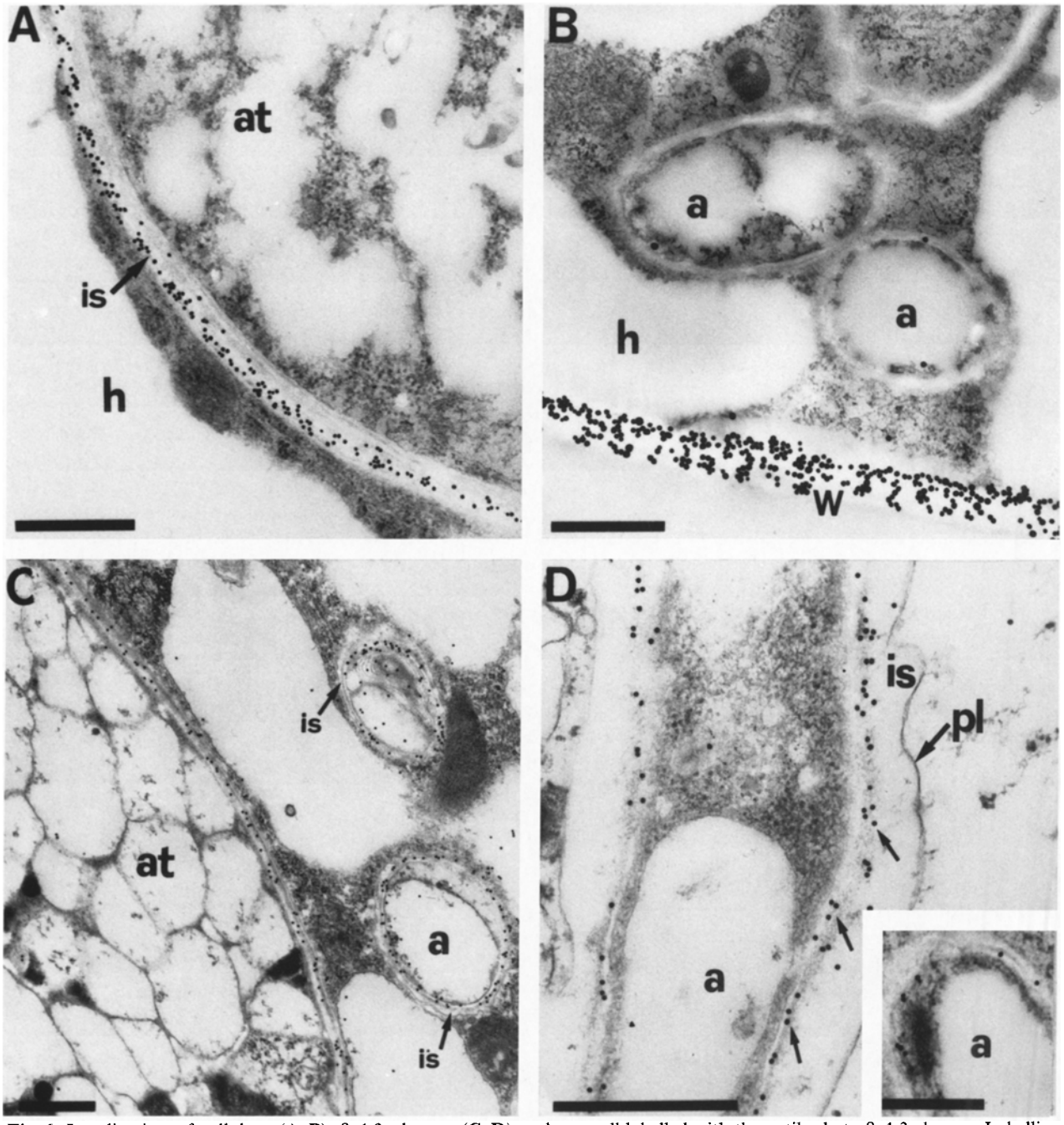

Fig. 6. Localization of cellulose (**A–B**), β–1,3 glucans (**C–D**) and chitin (*inset*) in ultra-thin sections of a mycorrhizal root. **A** After treatment with CBH I-gold complex, gold granules are present in the interface space around the large intracellular arbuscular branches (*at*). × 36000. **B** Only a few granules are present around the thin arbuscular branches. By contrast, the host plant wall is heavily labelled. × 36000. **C** General view of an arbuscule-infected cell labelled with the antibody to β–1,3 glucans. Labelling is particularly abundant over the fungal wall. × 24000. **D** Higher magnification of a fungal hypha following the same treatment. Labelling is limited to the fungal wall (*arrows*). × 58000. *Inset* The fungal wall is labelled after wheat-germ agglutinin-gold complex treatment. × 33000. *a*, arbuscule thin branches; *at*, arbuscule trunk; *h*, host; *is*, interface space; *pl*, plasmamembrane. Bars = 0.5 μm

belling was abundant at the penetration point, where there was continuity between the host wall and the interfacial area (Fig. 5B), as well as around the larger branches and the collapsed hyphae (Fig. 5C). The antibody also labelled the interface between the plant and the thin arbuscular branches (Fig. 5D,E), where the label closely adhered to the fungal wall. This wall was not labelled when the fungus was not in contact with the plant (not shown). The complex CBH I-gold adhered to cellulose over the plant wall and around the large intracellular branches. By contrast, only a limited number of granules were found around the thin arbuscular branches (Fig. 6A,B). When the β–1,3-polyglucopyranose antibody was used, the labelling of the maize cortical cells was limited to the rare plasmodemata (data not shown), while it was particularly abundant over the fungal walls

(Figs. 6C,D). No labelling was found in the interface zone. The fungal wall of the extraradical hyphae was strongly labelled (not shown). The fungal wall was always regularly labelled by wheat-germ agglutinin (Fig. 6D, inset).

Discussion

The use of specific cell-wall probes in labelling experiments reveals a similar location pattern of a maize HRGP and cellulose in the walls of both meristematic and differentiated parts of the roots. When the cortex is colonized by a symbiotic mycorrhizal fungus, HRGP and cellulose are expressed in two sites: the wall, and the interface area. In uninfected roots of the same age, however, they are only present in the inner part of the wall. Callose is not laid down at the interface between the plant and fungus, whereas it appears to be a skeletal component of the fungal wall.

Deposition of HRGP and cellulose in maize roots. The role of HRGPs as cell-surface glycoproteins involved in developmental processes increasingly attracts attention. Their expression is regulated during the maize embryogenesis (Ruiz-Avila et al. 1992) and during the acquisition of anatomical complexity in carrot roots (Smallwood et al.1994). The HRGPs have been localized in the walls of the root cell types so far examined (root cap, epidermal cortical and central cyclinder cells) according to Stafstrom and Stahelin (1988), Bonfante et al. (1991) and Smallwood et al. (1994). The conspicuous presence of HRGP in the walls of the root apex, observed in the present study, offers further evidence of the high HRGP transcriptional activity found in tissues with mitotic activity (Ludevid et al. 1990). In addition, our results provide new information on the relation between HRGP and cellulose. The distribution pattern of the polysaccharide is similar to that of HRGP in the cell of dividing cells. Both are spread over the entire newly deposited walls, but concentrated in more-specific sites (near the plasmamembrane, or in the inner part of the wall, with the constant exclusion of the middle lamella), when the primary wall is fully differentiated. This suggests a close interaction between the two wall components in the formation of the basic architecture of the cell wall.

Host cell-wall components in the interface area in the mycorrhizal roots. In the mycorrhizal maize roots, HRGP and cellulose are found in the interface between the intracellular fungus and the host membrane. Their co-location in this compartment recalls the situation described in the newly laid-down walls of the meristematic cells. We suggest that a mycorrhizal cell is involved in the de novo synthesis of cell-wall-like material, and modifies its pattern of expression. Nuclear activation in the host plant following the fungal infection has already been suggested in leek, where structural and positional modifications have been described in the nucleus of infected cells (Berta et al. 1990; Balestrini et al. 1992).

The defence responses of maize: HRGP and β–1,3-glucan location in mycorrhizal roots. Wounding, pathogenic fungi or fungal elicitors induce genes coding for HRGPs, and cause an accumulation of HRGP transcripts (Showalter et al. 1985; Templeton et al. 1990). In maize, too, the HRGP gene is induced by wounding and ethylene (Ludevid et al. 1990; Tagu et al. 1992). Our results do not allow us to distinguish between HRGP depostion as a result of mechanisms related to those acting during cell division or those related to defence reactions. However, on the basis of the morphological features showing a high level of compatibility between the partners, and of the weak expression of proteins usually associated with activation of plant defence systems, (Bonfante and Perotto 1992; Lambais and Medhy 1993), HRGP accumulation in the interface area does not seem to be related to defence mechanisms. Another molecule which is a constitutive cell-wall component, but tends to increase in pathogenic and/or incompatible associations is callose. However, the β–1,3-glucans indicative of callose do not show any change in their location following fungal infection of maize roots. They are located in the plasmodemata, according to Northcote et al. (1989). The situation in maize is therefore different from that described in a non-mycorrhizal mutant of pea (Gollotte et al. 1993), where callose was located at the contact point beween the extra-radical hypha and the wall of the epidermal host cell, suggesting a specific host reaction. Our findings provide a clear demonstration that in mycorrhizal maize roots the β–1,3-glucans in the interface compartment are not a component produced by the host. By contrast, as already suggested by Gollotte et al. (1993), β–1,3-glucans are a skeletal component of the fungal wall, together with chitin (Bonfante et al. 1990a).

In conclusion, the interface established between the plant and the symbiotic fungus may be regarded as a new compartment, typical of the symbiotic status. Its formation is the consequence of the induction of many mechanisms, including those involving the synthesis of cell-wall components. It may be suggested that they are mechanisms normally at work in the early stages of cell life, and open to reactivation by the establishment of the mycorrhizal symbiosis.

The research was supported by the Italian Murst (40%) grant and by an International Project between Spain and Italy (Azioni Integrate).

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