Mycorrhiza (1995) 5:157–163

ORIGINAL PAPER

Renato Peretto · Vittorio Bettini · Francesco Favaron
Paolo Alghisi · Paola Bonfante

Polygalacturonase activity and location in arbuscular mycorrhizal roots of *Allium porrum* L.

Abstract Polygalacturonase activity and location were analysed in leek roots (*Allium porrum* L.) colonized by *Glomus versiforme* (Karst.) Berch, an arbuscular mycorrhizal (AM) fungus. Polygalacturonase activity in mycorrhizal roots did not differ quantitatively from that found in nonmycorrhizal roots on all of the four harvesting dates. Fractionation of mycorrhizal root extracts by ion-exchange chromatography showed that expression of polygalacturonase was specific to the mutualistic association. Immunofluorescence and immunogold experiments were carried out to locate the polygalacturonase in mycorrhizal roots using a polyclonal antibody raised against a *Fusarium moniliforme* endopolygalacturonase. Immunolabelling was observed all over the arbuscules (intracellular fungal structures) but particularly at the interface between the arbuscule and the plant membrane. Since pectins are located in this area, we suggest that polygalacturonase produced during the symbiosis could play a role in plant pectin degradation.

Key words *Allium porrum* · Arbuscular mycorrhiza
Pectin · Polygalacturonase · Immunolocation

Introduction

The Glomales comprise a few symbiotic arbuscular mycorrhizal (AM) fungi that colonize the roots of about 80% of all plant species (Walker 1992). Despite this impressive biodiversity, the infection process is very constant. Penetrating hyphae from appressoria produced by the fungus colonize the root by means of coils, intercellular hyphae, vesicles and arbuscules which infect epidermal and cortical cells (Bonfante 1984). Fungal morphology during infection has been described in detail but the molecular mechanisms underlying the fungal-plant interactions are mostly unknown (Bonfante and Perotto 1992). Plant defence responses are scarcely activated by AM fungi (Dumas et al. 1992; Lambais and Mehdy 1993), despite colonization of as much as 60–80% of the entire root system.

Our working hypothesis is that the high degree of compatibility between the symbionts is partly the result of regulated production of cell wall hydrolytic enzymes, particularly the polygalacturonases. Polygalacturonases produced by a phytopathogenic fungus are important determinants of its pathogenicity, since they allow it to colonize plant tissues and obtain food. On the other hand, they give rise to oligogalacturonides, which activate plant defence mechanisms (Hahn et al. 1989). Both arbuscular and ericoid mycorrhizal fungi possess pectinolytic activities (Garcia-Romera et al. 1991a, b; Peretto et al. 1993), but their role in the establishment of the symbiosis has not been investigated.

This paper compares polygalacturonase activity in the uninfected and *Glomus versiforme*-colonized roots of *Allium porrum*, and describes the location of the enzyme by means of immunofluorescence and immunogold techniques.

R. Peretto · P. Bonfante (✉)
Dipartimento di Biologia Vegetale dell'Università,
Viale Mattioli, 25, I-10125 Turin, Italy
Fax: (11) 655839

V. Bettini · P. Bonfante
Centro di Studio Sulla Micologia del Terreno, CNR,
Viale Mattioli, 25, I-10125 Turin, Italy

F. Favaron · P. Alghisi
Istituto di Patologia Vegetale, Università di Padova,
Via Gradenigo, 6, I-35131 Padua, Italy

Materials and methods

Plant material

Seeds of *A. porrum* L., cv. "Mostruoso di Carentan" (Sementi Dotto, Mortegliano, Udine, Italy) were sown in pots of sterilized quartz sand and watered every other day with low-phosphorus (3.2 μM Na_2NPO_4) Long Ashton solution. A spore suspension obtained from *G. versiforme* (Karst.) Berch fruitbodies was injected around the 2-week-old seedlings to establish mycorrhizal

infection. The percentage of total infected root length was evaluated by the gridline intersect method of Giovannetti and Mosse (1980).

Root protein extraction

Roots were harvested from 30-, 48-, 70- and 105-day-old plants, cut below the crown. Samples (5 g fresh wt. for each harvesting date) were frozen with liquid nitrogen and crushed with a chilled (−20° C) mortar and pestle in the presence of insoluble polyvinylpyrrolidone (10% root fresh wt.). The powder was suspended in the cold extraction buffer (approximately 1 ml/g root fresh wt.) consisting of 50 mM Tris-HCl (pH 7.5), 2.5 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 0.5 M NaCl and 10% (v/v) glycerol. After 2 h on ice, cell debris was removed by filtration with cheesecloth, centrifugation at 4000 *g* for 20 min and ultracentrifugation at 20000 *g* for 30 min. The solutions were dialysed overnight against 20 mM sodium acetate buffer (pH 5) and 0.15 M NaCl and assayed for polygalacturonase activity.

Protein concentration was measured according to Bradford (1976) using the BioRad (Richmond, Calif.) dye reagent and bovine serum albumin (Sigma, St. Louis, Mo.) as a standard.

Polygalacturonase assay

Polygalacturonase activity was determined by measuring the increase in reducing end-groups according to the method of Milner and Avigad (1967). Reaction mixtures contained 0.1 ml of sample and 0.1 ml of 0.5% polygalacturonic acid (Fluka, Buchs, Switzerland) dissolved in 50 mM sodium acetate buffer (pH 5), 0.15 M NaCl. They were incubated at 30° C for 6 h. One unit of polygalacturonase activity was defined as the amount of enzyme producing 1 μmol of reducing end-groups/min.

Crude extract fractionation

Crude protein extracts from 30 g of mycorrhizal and nonmycorrhizal 80-day-old roots were fractionated by fast protein liquid chromatography (FPLC). The samples, adjusted to pH 7.8 by 20 mM Tris-HCl buffer, were loaded onto a strong anion exchange column (Mono Q; Pharmacia LKB Biotech., Uppsala, Sweden) equilibrated with 20 mM Tris-HCl buffer (pH 7.8). The flow-through volumes were then applied to a strong cation exchange column (Mono S; Pharmacia) adjusted to pH 4.2 by acetic acid. The Mono Q and Mono S columns were eluted with 30 ml of a linear NaCl gradient (0–0.5 M). Fractions of 0.75 ml were collected and assayed for polygalacturonase activity.

Microscopy and immunocytochemistry

Immunolabelling experiments were performed by using a polyclonal antibody raised against *Fusarium moniliforme* endopolygalacturonase (De Lorenzo et al. 1987).

For electron microscopy, root segments from infected and unifected roots were fixed in 2.5% glutaraldehyde and then embedded in LR White resin as described in Peretto et al. (1992). Thin sections were incubated with the anti-endopolygalacturonase antibody (diluted 1:1000), followed by goat anti-rabbit gold conjugate (15 nm particles, Bio Cell, Cardiff, UK) according to Bonfante et al. (1990b).

For immunofluorescence, root segments were fixed in MTSB (4% p-formaldehyde in 50 mM Pipes pH 6.9, 5 mM $MgSO_4$, 5 mM EGTA) 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 MTSB. The external mycelium was collected from the root surface, washed several times with water and fixed in MTSM for 2 h. Root sections 30–40 μm thick were obtained with a cryomicrotome (Microm, Heidelberg, Germany). Sections and external mycelium were immunolabelled as described in Peretto et al. (1992). The primary antibody was diluted 1:200. The secondary anti-rabbit antibody (diluted 1:50) was rhodamine conjugated (Sigma), since no autofluorescence was detected at 546 nm in the sample sections. Controls were performed by omitting the primary antibody step.

Some mycorrhizal root sections were simultaneously treated with wheat germ agglutinin (WGA), a lectin that specifically binds to *N*-acetylglucosamine, according to Bonfante et al. (1990a); WGA was conjugated to FITC (fluorescein isothiocyanate).

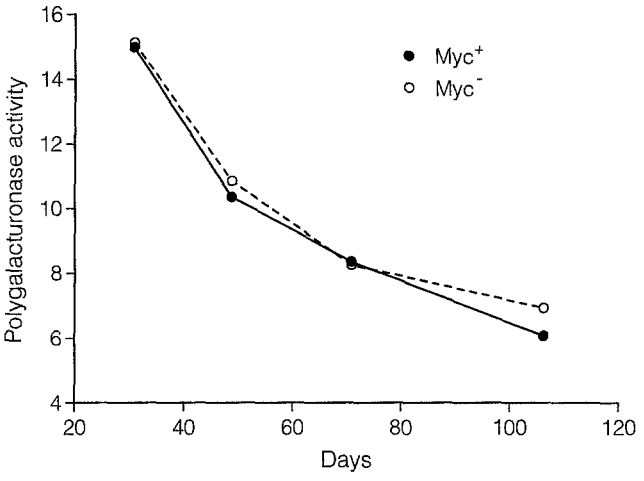


Fig. 1 Polygalacturonase activity detected in crude extracts of mycorrhizal (*Myc*$^+$) and nonmycorrhizal (*Myc*$^-$) *Allium porrum* roots during plant growth. Activity is expressed as RGU·10^{-4}/g root fresh wt., where RGU is the amount of enzyme producing 1 μmol of reducing end groups/min

Results

Evaluation of polygalacturonase activity in mycorrhizal and nonmycorrhizal *A. porrum* roots

Polygalacturonase activity in crude extracts of both mycorrhizal and nonmycorrhizal roots was very low. It always decreased with plant age and no significant differences between the samples were observed (Fig. 1).

FPLC analysis of polygalacturonase activity

Crude extracts from 80-day-old mycorrhizal and nonmycorrhizal roots were fractionated by FPLC. The percentage infection in mycorrhizal roots was about 64%. Passage through a Mono Q column revealed two peaks of polygalacturonase activity (Fig. 2) at about 0.12 M NaCl (peak I) and 0.3 M NaCl (peak II) in both root types.

The flow-through volumes containing activity not retained in the Mono Q column were applied to a Mono S column. A new peak was found at about 0.16 M NaCl (peak III) in both samples (Fig. 3). Two additional peaks at about 0.18 M NaCl (peak IV) and 0.3 M NaCl (peak V) were detected in the mycorrhizal

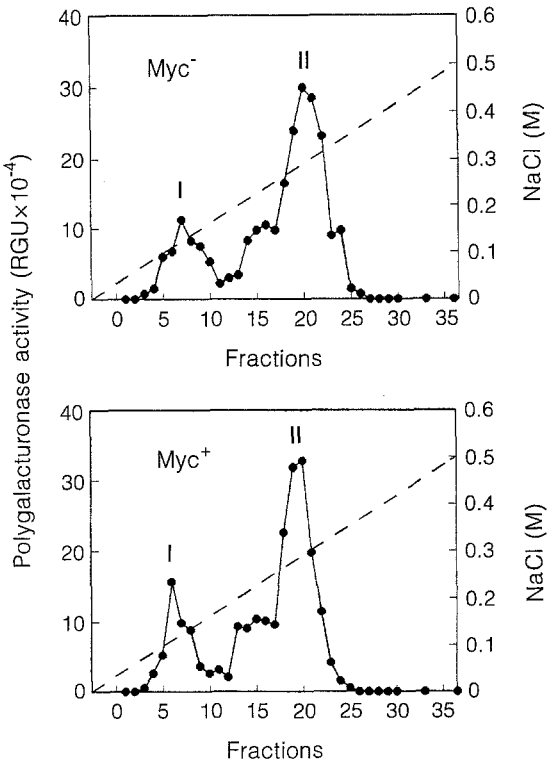


Fig. 2 Polygalacturonase activity in Mono Q fractions derived from extracts of 80-day-old mycorrhizal (*Myc*$^+$) and nonmycorrhizal (*Myc*$^-$) *A. porrum* roots. About $250 \cdot 10^{-4}$ RGU of each sample were loaded onto the column. *Dashed line* NaCl gradient

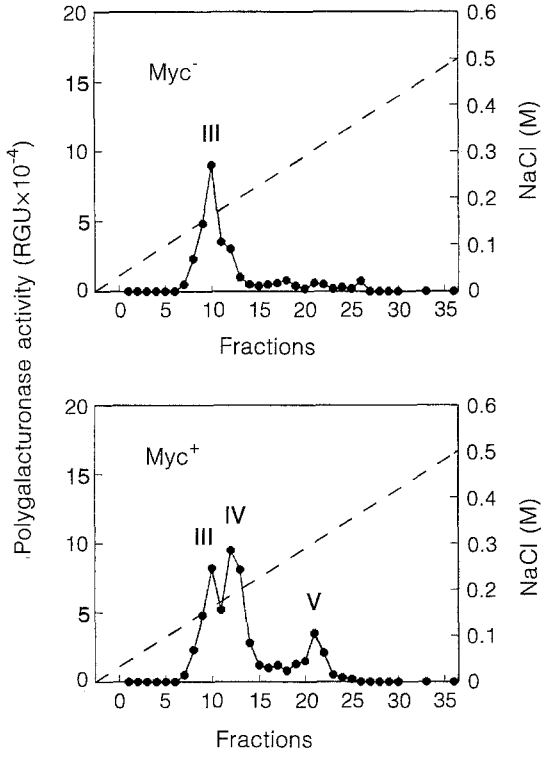


Fig. 3 Polygalacturonase activity in Mono S fractions. The flow-through volumes from the Mono Q column were loaded onto the Mono S column. *Dashed line* NaCl gradient

sample only (Fig. 3), representing polygalacturonases expressed solely during the symbiotic phase.

Immunolocation of polygalacturonase

Extraradical hyphae of *G. versiforme* running over the root surface were irregularly fluorescent after treatment with the anti-endopolygalacturonase antibody and the secondary rhodamine-conjugated antibody (Fig. 4). No fluorescence was detected with the secondary antibody alone.

During infection (for morphological details see Bonfante et al. 1990a), the fungus produces large intercellular hyphae (Fig. 5). No labelling was observed in these hyphae after the immunofluorescence experiments on the frozen sections (Fig. 6). In contrast, substantial labelling was always observed at the centre of arbuscule-infected cells, as a bag-like structure without a well-defined profile (Fig. 7). Simultaneous labelling with the lectin WGA-FITC, which precisely stained the wall of the fungal branches, clearly revealed that the immunolabelling corresponded to the area occupied by the arbuscule (Fig. 8); the penetration point (Fig. 8, inset) was also labelled. No labelling was found in the control sections where the primary antibody was omitted.

Immunogold experiments on thin sections from mycorrhizal roots showed gold granules over the wall of the extraradical hyphae (results not shown), confirming the immunofluorescence results (Fig. 4). During the intraradical phase, granules were only found in the arbuscule-infected cells. Labelling was regularly present in the space between the arbuscular branches and the invaginated plant cell membrane. It was loose around the larger branches (Fig. 9) and denser around the thin arbuscular branches (Fig. 10). Some gold granules were also observed in the fungus cytoplasm (Fig. 10). A glancing section of a thin branch showed the occurrence of gold granules over the fungal wall (Fig. 11). No labelling was found on the root tissues or in the differentiated areas of the unifected roots (results not shown), where the labelling was limited to the meristems of the emerging lateral roots (Peretto et al. 1992). No granules were observed in the control experiments (Fig. 12).

Discussion

Polygalacturonase production in mycorrhizal *A. porrum* roots

Differences in polygalacturonase activity in mycorrhizal and nonmycorrhizal roots of *A. porrum* do not provide evidence of quantitative changes following establishment of the symbiosis. The activity expressed in nonmycorrhizal roots has been related to emission of lateral roots in young *A. porrum* seedlings (Peretto et al. 1992). In the present experiments, activity decreased

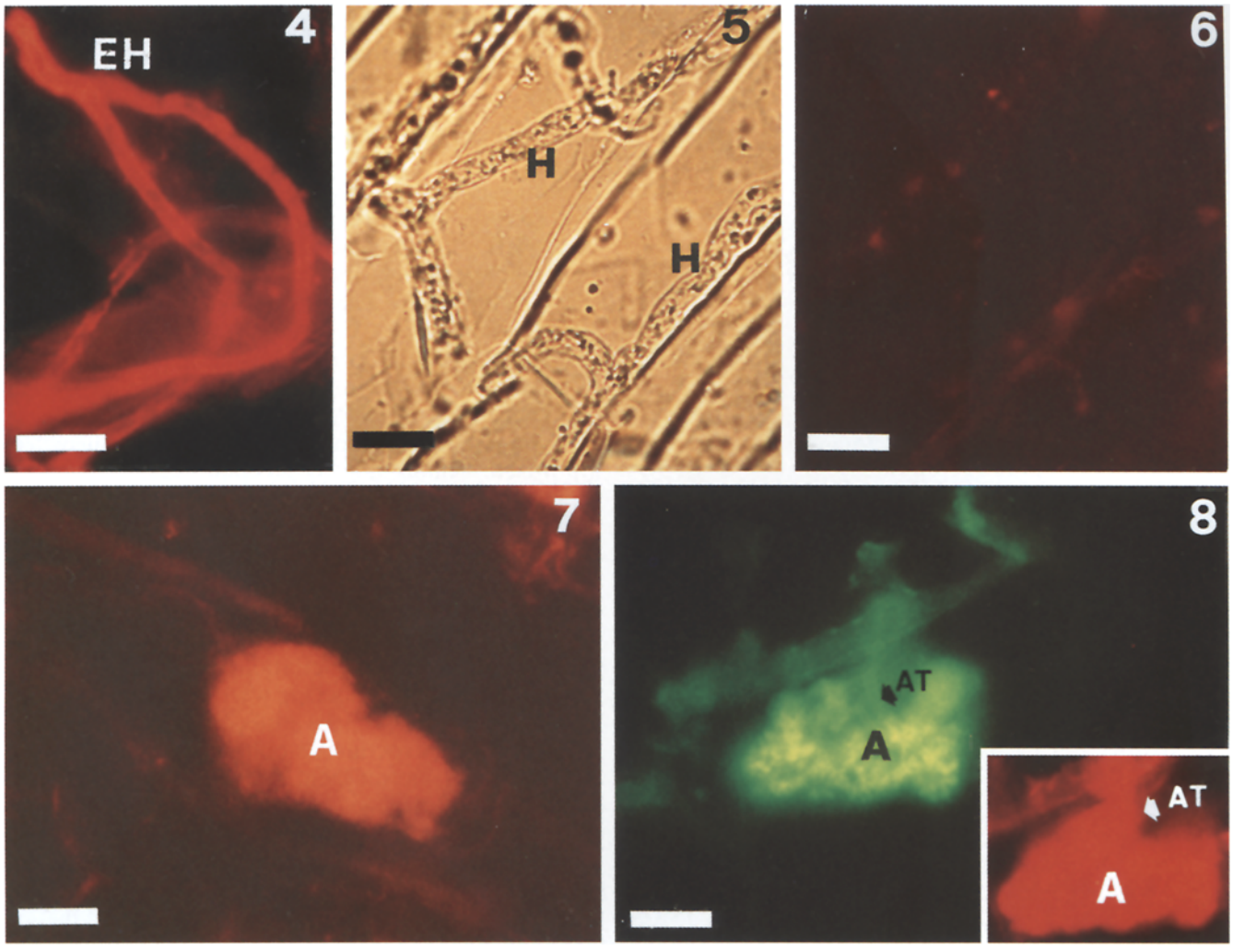


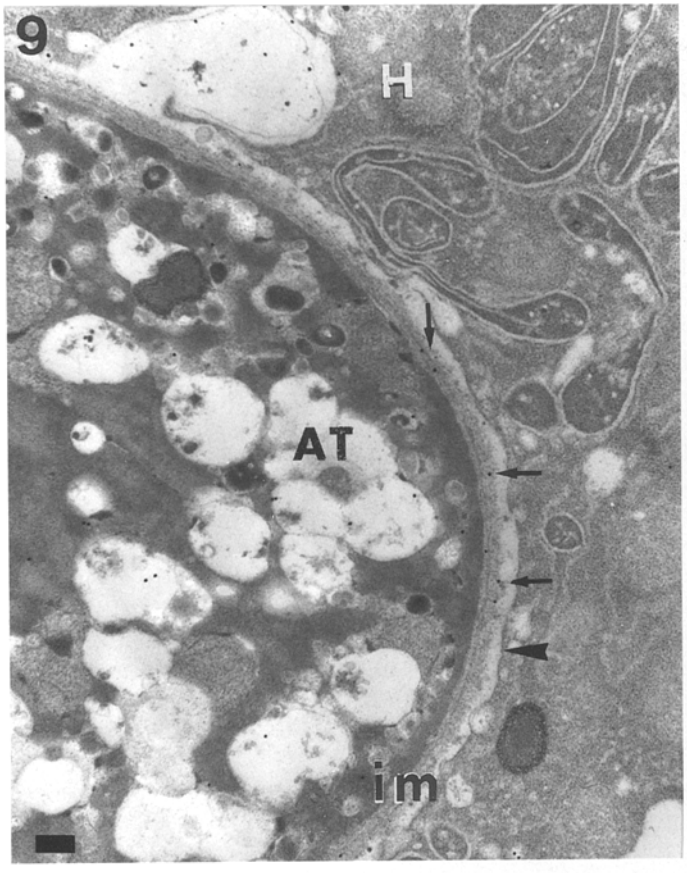
Fig. 4 External hyphae (*EH*) of *Glomus versiforme* seen under a fluorescence microscope. The mycelium was treated with a polyclonal antibody raised against *Fusarium moniliforme* endopolygalacturonase and then labelled with a rhodamine-conjugated secondary antibody; *bar* 20 μm

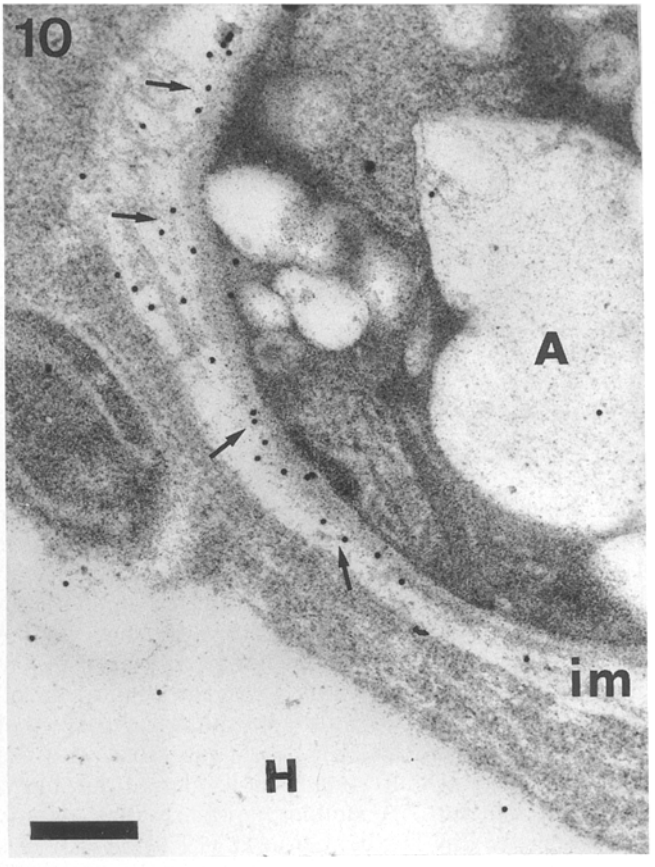
Figs. 5–8 Frozen sections of mycorrhizal *A. porrum* roots. Sections were treated with a polyclonal antibody raised against *F. moniliforme* endopolygalacturonase and then labelled with a rhodamine-conjugated secondary antibody; *bars* 20 μm. **Fig. 5** Large fungal hyphae (*H*) running between host cortical cells. **Fig. 6** The same section as in Fig. 5 under a fluorescence microscope. No labelling is evident over the hyphae or over the cortical cells. **Fig. 7** The arbuscule (*A*) inside a root cell is strongly labelled. **Fig. 8** The labelling of the arbuscule (*A*) with fluorescein isothiocyanate-conjugated wheat germ agglutinin reveals the arbuscule cell walls more precisely than the antibody labelling (*inset*). This seems to label the area limited by the periarbuscular membrane. The arbuscular trunk (*AT*) corresponding to the penetration point is also immunolabelled (*inset*)

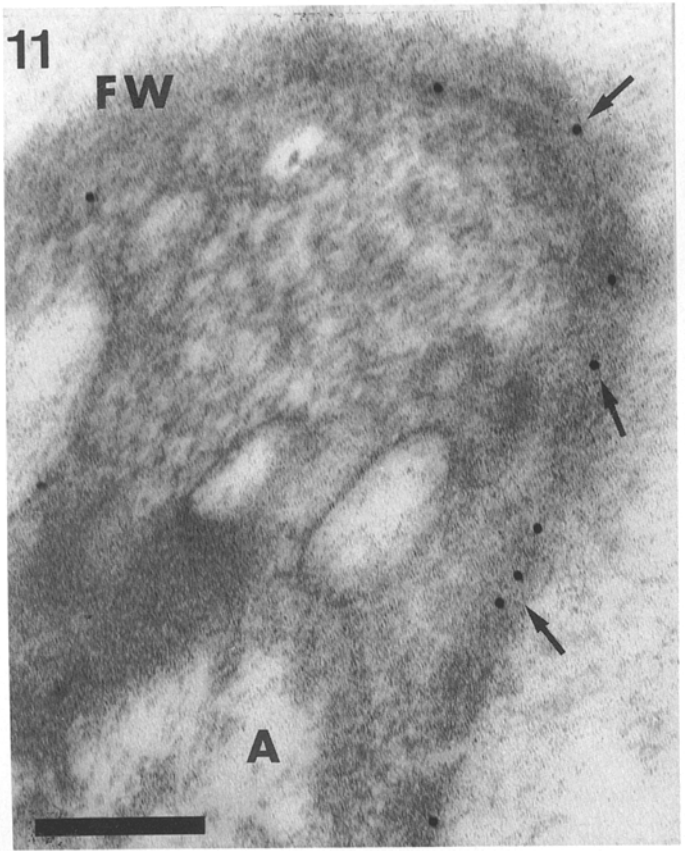
with time both in mycorrhizal and nonmycorrhizal roots, following a pattern corresponding to that shown for the association *G. mosseae*/lettuce (Garcia-Romera et al. 1991b). A qualitative difference was found by FPLC analysis in the form of two additional polygalacturonase peaks in the mycorrhizal sample.

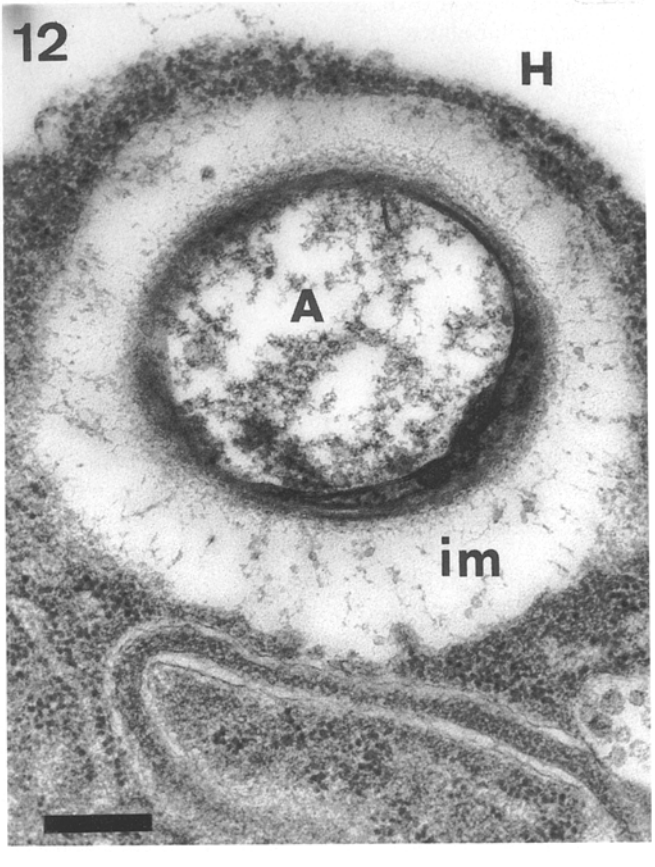
It may thus be suggested that polygalacturonases specific for the symbiotic phase are expressed in the mycorrhizal roots, and that they are produced by *G. versiforme*. A related species, *G. mosseae*, has already been found to produce various pectinolytic enzymes, including polygalacturonases, during its sporal and extraradical phase (Garcia-Romera et al. 1991a).

Figs. 9–12 Transmission electron micrographs of mycorrhizal *A. porrum* roots; *bars* 0.25 μm. **Fig. 9** The interface material (*im*) between a large arbuscular trunk (*AT*) and the host cell (*H*) displays only few gold granules (*arrows*) after the immunogold labelling to reveal polygalacturonase. Sections were treated with a polyglonal antibody raised against *F. moniliforme* endopolygalacturonase and then labelled with a gold-conjugated secondary antibody. The *arrowhead* points to the root cell membrane. **Fig. 10** Interface area between a thin arbuscule branch (*A*) and the host cell (*H*). The immunogold labelling with the anti-polygalacturonase antibody produced a dense distribution of gold granules (*arrows*) over the interfacial material (*im*). **Fig. 11** Glancing section of a thin arbuscular branch (*A*). The location of gold granules (*arrows*) over the fungal wall (*FW*) is evident at high magnification. **Fig. 12** Control experiment. After omitting the primary antibody step, no gold granules were found in the interface zone (*A* arbuscule, *H* host cell, *im* interfacial material) ▶


9
H
AT
im


10
A
im
H


11
FW
A


12
H
A
im

In situ immunolabelling provides more direct experimental evidence of the fungal nature of the polygalacturonase. The two symbiosis-related enzymes could not be purified due to their very low production. Therefore, an antibody raised against a *F. moniliforme* endopolygalacturonase (De Lorenzo et al. 1987) was used as a probe, since polygalacturonases from bacteria, fungi and plants share several regions of homology in their amino acid sequences (Caprari et al. 1993); this allowed us to use an heterologous antibody to reveal the symbiosis-related polygalacturonase. The gold granules were located over the fungal cytoplasm and the fungal wall and in the interface area. This means that the enzyme(s) produced by the fungus is released to the outside and freely diffuses into the interface space limited by the host membrane, leading to the bag-like pattern observed in the immunofluorescence experiments. In the interface compartment, nonesterified pectins have been located together with other cell wall molecules of host origin (Bonfante et al. 1990b). The simultaneous presence of the enzyme and its substrate suggests that the fungus can use the pectins newly laid down by the plant as a source of food during its intraradical development. This could explain the morphological aspect of the interfacial material, which is continuous with the host cell wall and shares many molecules with it, but is looser in structure. A similar hypothesis has already been suggested by Dexheimer et al. (1979) on the basis of morphological evidence.

Immunolabelling of the external hyphae suggests that fungal polygalacturonase, if active, may play some role in the exploitation of organic molecules free in the soil. By contrast, the lack of labelling in the intercellular hyphae could be related to the presence of wide air channels (Brundrett and Kendrick 1990) in the roots; in this case, fungal colonization would not require enzymatic activity.

Polygalacturonase expression in mycorrhizal versus pathogenic fungi

Previous morphological observations demonstrated that only limited and subtle changes are produced by *G. versiforme* in the host cell wall during penetration (Bonfante and Vian 1989). These data, together with the present enzymatic and immunological results, suggest that production of the fungal polygalacturonase(s) is kept at low levels and is located at very precise sites during symbiosis. This pattern may be part of the infection strategy of AM fungi, by which they maintain the viability of the host and avoid its defence reactions.

This mechanism presents interesting similarities to the infection strategy of phytopathogenic fungi, in particular obligate biotrophs. Necrotrophic fungi produce high amounts of polygalacturonases and these are considered to be markers of pathogenicity or virulence (Collmer and Keen 1986). By contrast, biotrophic fungi are characterized by low and regulated production of cell-wall-degrading enzymes, mainly cellulases and pectinolytic enzymes such as pectin esterase and polygalacturonate lyase (Deising and Mendgen 1992; review, Mendgen and Deising 1993). Our results, therefore, support the hypothesis that AM fungi, like the other biotrophic fungi, produce only low pectinolytic activities (Garcia-Romera et al. 1991a, b).

In plant-fungal interactions, evidence for the role of fungal cell-wall-degrading enzymes has been mostly derived from ultrastructural observations of plant cell wall degradation (O'Connell and Bailey 1991). Only in the hemibiotrophic fungus *Colletotrichum lindemuthianum* was endopolygalacturonase detected on the fungal cell wall and at the interface with the host, suggesting that the fungus causes drastic degradation of the pectic material of primary walls (Benhamou et al. 1991). However, in this case an indirect probe was used, since the endopolygalacturonase was revealed with a polygalacturonase-inhibiting protein (PGIP) bound to the gold. It was assumed that the PGIP binds to its substrate (the fungal polygalacturonase) and so reveals the sites of accumulation. In our present experiments, an antibody against a fungal endopolygalacturonase allowed direct detection of the site of the antigen expression.

In conclusion, the present results point to a low and regulated production of polygalacturonases during the symbiosis, the origin of which can be traced to the fungus. Enzyme activity is quantitatively similar to that in nonmycorrhizal roots, since the additional enzymes related to the activity peaks in the mycorrhizal roots seem to be only expressed during the arbuscular phase. This raises the questions of whether the enzymes are substrate inducible, whether other cell wall hydrolytic enzymes are involved, and/or whether mechanical forces are more important in the other symbiotic steps.

Acknowledgements The authors are grateful to Professor F. Cervone (Dipartimento di Biologia Vegetale, Università di Roma "La Sapienza", Rome, Italy) for kindly providing the polyclonal antibody raised against the *Fusarium moniliforme* endopolygalacturonase. This research was supported by the National Research Council of Italy, Special Project RAISA, Subproject No. 2, No. 1510.

References

Benhamou N, Lafitte C, Barthe J-P, Esquerré-Tugayé MT (1991) Cell surface interactions between bean leaf cells and *Colletotrichum lindemuthianum*. Cytochemical aspects of pectin breakdown and fungal endopolygalacturonase accumulation. Plant Physiol 97:234–244

Bonfante P (1984) Anatomy and morphology. In: Powell CL, Bagyaraj DJ (eds) VA Mycorrhizas. CRC Press, Boca Raton, Fla, pp 5–33

Bonfante P, Perotto S (1992) Plants and endomycorrhizal fungi: the cellular and molecular basis of their interaction. In: Verma DPS (ed) Molecular signals in plant-microbe communications. CRC Press, Boca Raton, Fla, pp 445–470

Bonfante P, Vian B (1989) Cell wall texture in symbiotic roots of *Allium porrum* L. Ann Sci Nat Bot Biol Veg 10:97–109

Bonfante P, Faccio A, Perotto S, Schubert A (1990a) Correlation between chitin distribution and cell wall morphology in the mycorrhizal fungus *Glomus versiforme*. Mycol Res 94:157–165

Bonfante P, Vian B, Perotto S, Faccio A, Knox JP (1990b) Cellulose and pectin localization in roots of mycorrhizal *Allium porrum*: labelling continuity between host cell wall and interfacial material. Planta 180:537–547

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248

Brundrett MC, Kendrick B (1990) The roots and mycorrhizas of herbaceous woodland plants. II. Structural aspects of morphology. New Phytol 114:469–479

Caprari C, Richter A, Bergmann C, Lo Cicero S, Salvi G, Cervone F, De Lorenzo G (1993) Cloning and characterization of a gene encoding the endopolygalacturonase of *Fusarium moniliforme*. Mycol Res 97:497–505

Collmer A, Keen NT (1986) The role of pectic enzymes in plant pathogenesis. Annu Rev Phytopathol 24:383–409

Deising H, Mendgen K (1992) Developmental control of enzyme production and cell wall modification in rust fungi, and defence reactions of the host plant. In: Tudzynski P, Stahl U (eds) Molecular biology of filamentous fungi. VCH, Weinheim New York, pp 27–44

De Lorenzo G, Salvi G, Degrà R, D'Ovidio R, Cervone F (1987) Induction of extracellular polygalacturonase and its mRNA in the phytopathogenic fungus *Fusarium moniliforme*. J Gen Microbiol 133:3365–3373

Dexheimer J, Gianinazzi S, Gianinazzi-Person V (1979) Ultrastructural cytochemistry of the host-fungus interfaces in the endomycorrhizal association *Glomus mosseae/Allium cepa*. Z Pflanzenphysiol 92:191–206

Dumas-Gaudot E, Furlan V, Grenier J, Asselin A (1992) New acidic chitinase isoforms induced in tobacco roots by vesicular-arbuscular mycorrhizal fungi. Mycorrhiza 1:133–136

Garcia-Romera I, Garcia-Garrido JM, Ocampo JA (1991a) Pectolytic enzymes in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. FEMS Microbiol Lett 78:343–346

Garcia-Romera I, Garcia-Garrido JM, Ocampo JA (1991b) Pectinase activity in vesicular-arbuscular mycorrhiza during colonization of lettuce. Symbiosis 12:189–198

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. New Phytol 84:489–500

Hahn MG, Bucheli P, Cervone F, Doares SH, O'Neill RA, Darvill A, Albersheim P (1989) The role of cell wall constituents in plant-pathogen interactions. In: Nester E, Kosuge T (eds) Plant-microbe interactions. McGraw-Hill, New York, pp 131–181

Lambais MR, Mehdy MC (1993) Suppression of endochitinase, β-1,3-endoglucanase, and chalcone isomerase expression in bean vesicular-arbuscular mycorrhizal roots under different soil phosphate conditions. Mol Plant-Microbe Interactions 6:75–83

Mendgen K, Deising H (1993) Tansley Review No. 48. Infection structures of fungal plant pathogens–a cytological and physiological evaluation. New Phytol 124:193–213

Milner Y, Avigad G (1967) Colorimetric assay of hexuronic acids and some keto sugars. Carbohydr Res 4:359

O'Connell RJ, Bailey JA (1991) Hemibiotrophy in *Colletotrichum lindemuthianum*. In: Mendgen K, Lesemann D-E (eds) Electron microscopy of plant pathogens. Springer, Berlin Heidelberg New York, pp 211–222

Peretto R, Favaron F, Bettini V, De Lorenzo G, Marini S, Alghisi P, Cervone F, Bonfante P (1992) Expression and localization of polygalacturonase during the outgrowth of lateral roots in *Allium porrum* L. Planta 188:164–172

Peretto R, Bettini V, Bonfante P (1993) Evidence of two polygalacturonases produced by a mycorrhizal ericoid fungus during its saprophytic growth. FEMS Microbiol Lett 114:85–92

Walker C (1992) Systematics and taxonomy of the arbuscular endomycorrhizal fungi (Glomales). Agronomie 12:887–897