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Hydroxyproline-rich glycoprotein mRNA accumulation in maize root cells colonized by an arbuscular mycorrhizal fungus as revealed by in situ hybridization

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Summary. To determine whether the expression of cell wall related genes changes during the establishment of an arbuscular mycorrhizal symbiosis (AM), we studied the expression of a maize hydroxyproline-rich glycoprotein (HRGP) gene. In situ hybridization showed that, in differentiated cells of maize roots, mRNA accumulation corresponding to the gene encoding for HRGP was only found when the cells were colonized by the endomycorrhizal fungus *Glomus versiforme*.

Keywords: Arbuscular mycorrhiza; *Zea mays*; Hydroxyproline-rich glycoprotein; In situ hybridization; *Glomus versiforme*.

Introduction

Arbuscular mycorrhizal (AM) fungi are members of the class Zygomycetes, order Glomales: they colonize the roots of about 80% of land plants to form symbioses which play crucial roles in plant nutrition and health (Gianinazzi and Schüepp 1994). Morphological observations have shown that during colonization dramatic changes take place in the structure of the cortical host cell: the nucleus moves from the periphery to a central position, amyloplasts disappear, the central vacuole becomes fragmented, and a new compartment (the interface) is created by invagination of the host membrane around the fungus (Bonfante and Perotto 1995). In situ affinity probes, namely enzymes, lectins, and antibodies, have allowed us to characterize this compartment as a zone of high molecular complexity. Molecules common to the plant primary wall, such as β-1,4-glucans, non-esterified homogalacturonans, xyloglucans, proteins rich in hydroxyproline (HRGPs), and arabinogalactan-proteins have been located at the interface in many different plant/AM fungus combinations (Bonfante 1994, Balestrini et al. 1996).

Several strategies have been used to demonstrate that plant gene expression changes during the establishment of mycorrhizal symbiosis (Gianinazzi-Pearson et al. 1995, Volpin et al. 1995, Harrison 1996). Even so, little is known about the molecular events involved in the creation of the interface during the colonization of roots by AM fungi. The presence of HRGPs in mycorrhizal roots is of particular interest: HRGPs are considered to be markers for both development, since they are strongly expressed in the meristem and during formation of lateral and adventitious roots (Stiefel et al. 1990, Ruiz-Avila et al. 1991, Vera et al. 1994), and stress, since their expression increases after pathogenic infection (Esquerrè-Tugayè et al. 1979, Wycoff et al. 1995) or wounding (Ludevid et al. 1990). In addition, labelling experiments have also located a maize HRGP in the interface area (Balestrini et al. 1994). These results open the question whether the HRGP located around the fungus is a new deposit or derived from existing, but not yet cell-wall bound, HRGP.

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This paper describes the mRNA accumulation patterns corresponding to a gene encoding a maize HRGP in the meristematic and differentiated parts of maize roots in the presence or absence of the mycorrhizal fungus *Glomus versiforme* as revealed by in situ hybridization.

Material and methods

Plant material

Seeds of *Zea mays* L. cv W64A obtained from plants grown in a greenhouse at Centro de Investigación y Desarollo, C.S.I.C., Barcelona were sown in sterilized quartz sand. The germinated seedlings were watered three times a week with low-phosphorus (6 mg of HPO_4^{2-} per l) Long Ashton solution (Hewitt 1966). Mycorrhizal plants were obtained by inoculating seedlings with a spore suspension of *Glomus versiforme* (Karst) Berch (BEG number 47). Original spores were provided by Dr. J. Trappe, Oregon State University, Corvallis, OR, U.S.A. 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.

Fixation and embedding of material

Apical and differentiated segments from uninfected and mycorrhizal roots were fixed in 4% paraformaldehyde in PBS (phosphate buffered saline: 130 mM NaCl; 7 mM Na_2HPO_4; 3 mM NaH_2PO_4, pH 7.4) overnight at room temperature. For the first 15–30 min, samples were fixed under vacuum to facilitate infiltration with the fixative. Thereafter, fixative solution was removed by washing in saline solution (150 mM NaCl) for 15 min at room temperature. The tissues were dehydrated successively in solutions of 30, 50, 70, 80, 95 (in 150 mM NaCl), 100% ethanol and 100% xylene for 30–60 min each step and embedded in paraffin wax (Paraplast plus; Sigma, Milan, Italy) at 60 °C. 7–8 μm sections were then transferred to slides treated with poly-L-lysine, 100 μg/ml (Sigma), and dried on a warming plate at 40 °C overnight.

Production of riboprobes

Digoxygenin (DIG)-labelled or ^{35}S-labelled RNA probes were synthesized starting with 1 μg of linearized template (Langdale 1993). DIG-labelled riboprobes (antisense and sense probes) were produced with digoxygenin-UTP by in vitro transcription using the bluescript T3 and T7 promoters according to the manufacturer's protocol (RNA labelling kit, Boehringer Mannheim, Mannheim, Federal Republic of Germany). ^{35}S-labelled probes were produced with ^{35}S-CTP (37 TBq/mmol; Amersham International, U.K.) according to the manufacturers.

In situ hybridization and detection

DIG-labelled antisense and sense transcripts were hybridized to sections as described in Torres et al. (1995). The sections were deparaffinized in xylene, rehydrated through an ethanol series, treated with 0.2 M HCl for 20 min, washed in sterile water for 5 min, incubated in 2× SSC for 10 min, washed in sterile water for 5 min, incubated with proteinase K (1 μg/ml in 100 mM Tris-HCl, pH 8.0, 50 mM EDTA; Boehringer Mannheim) at 37 °C for 30 min, washed briefly in PBS and then treated with 0.2% glycine in PBS for 5 min. After two rinses in PBS, slides were then incubated in 4% paraformaldehyde in PBS for 20 min, washed in PBS (2 times 5 min) and dehydrated in an ethanol series from 30 to 100%. Hybridization overnight at 55 °C was with denatured digoxygenin-labelled RNA probes in 50% formamide, 6× SSC, 3% SDS, 100 μg/ml tRNA, 100 μg/ml poly A. After hybridization, slides were washed twice in 1× SSC, 0.1% SDS at room temperature and rinsed with 0.2× SSC, 0.1% SDS at 55 °C (2 times 10 min). After rinsing with 2× SSC for 5 min at room temperature, the non-specifically bound DIG-labelled probes were removed by incubating in 10 μg of RNase A per ml in 2× SSC at 37 °C for 30 min. Slides were then rinsed twice in 2× SSC before proceeding to the next stage. The hybridized probes were detected using an alkaline phosphatase antibody conjugate (Boehringer Mannheim). After rinsing in TBS (100 mM Tris-HCl, pH 7.5, 400 mM NaCl) for 5 min, slides were treated with 0.5% blocking reagent in TBS for 1 h, incubated for 2 h with the anti-digoxygenin alkaline phosphatase conjugate diluted 1:500 in 0.5% BSA Fraction V in TBS and then washed in TBS (3 times 5 min). Colour development was carried out according to Torres et al. (1995). The colour reaction was stopped by washing in water and the sections were then dehydrated through an ethanol series, xylene and mounted in Histovitrex (Carlo Erba).

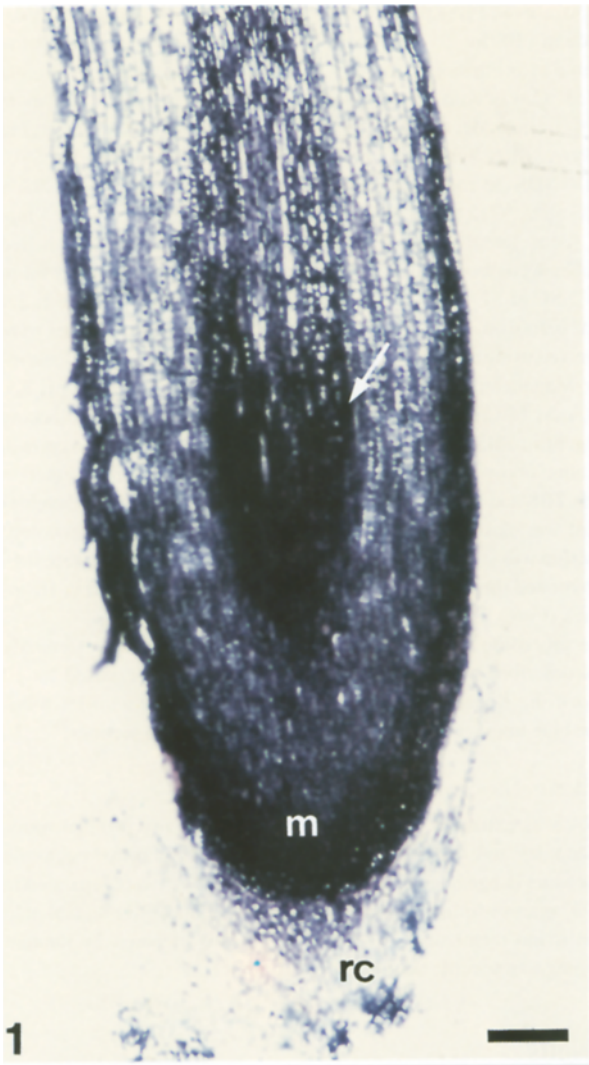
The procedure for radioactive in situ hybridization was essentially that described by Langdale (1993). The slides were exposed for 10 days using Kodak NTB-2 emulsion and then stained with 1% toluidine blue in order to examine the morphology of the sections.

RNA extraction and Northern blot analysis

RNA was extracted from mycorrhizal and non-mycorrhizal roots, coleoptiles and 4-day-old roots by the guanidinium-hydrochloride procedure (Logemann et al. 1987). The total RNAs were separated in 1.5% agarose-formaldehyde gel (Lehrach et al. 1977) and blotted onto nylon membrane. ^{32}P cDNA probe was prepared by random priming to a specific activity of 10^8 cpm/mg.

Results

RNA blot analysis revealed no change in the population of mRNA transcripts encoding for the maize HRGP (result not shown). To investigate whether the presence of the mycorrhizal fungus would activate the HRGP gene in specific cells, in situ hybridization experiments were carried out on paraffin sections of maize roots in apical and differentiated regions by using a genomic fragment of HRGP gene (600 bp long) as a probe. A DIG-labelled RNA antisense probe was followed by a hybridization signal that was more intense in meristematic and vascular differentiation regions (Fig. 1). The differentiated cap cells were not labelled as well as the already differentiated cortical cells (Fig. 1). This result agrees with previous in situ hybridization experiments showing a high HRGP transcriptional activity in tissues with mitotic activity and during vascular differentiation (Ludevid et al. 1990, Stiefel et al. 1990). The DIG-labelled RNA sense probe did not hybridize and after long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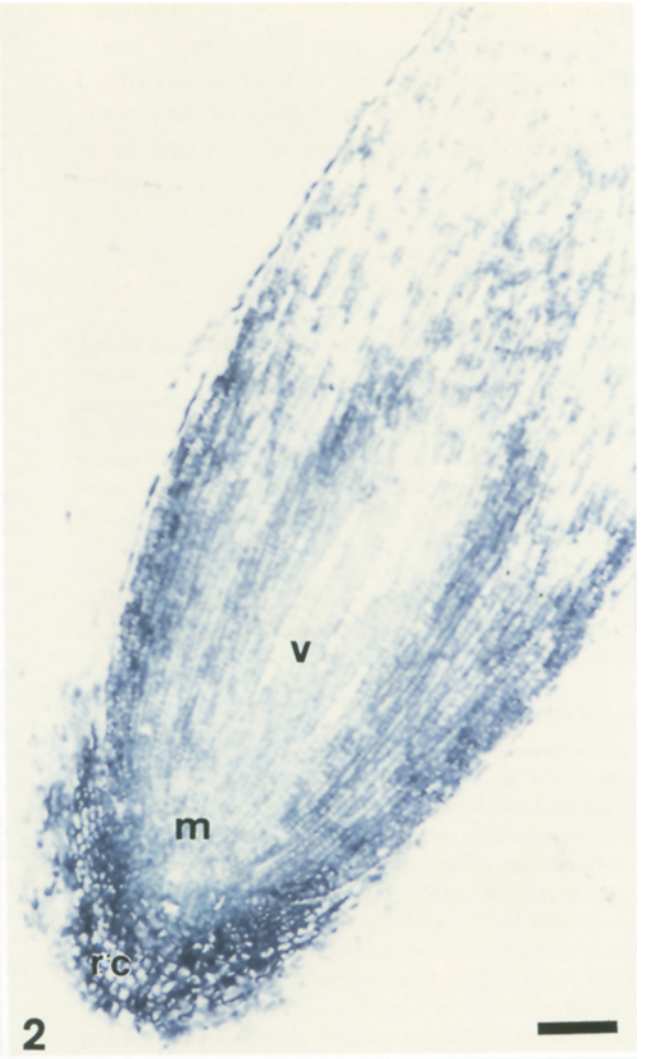
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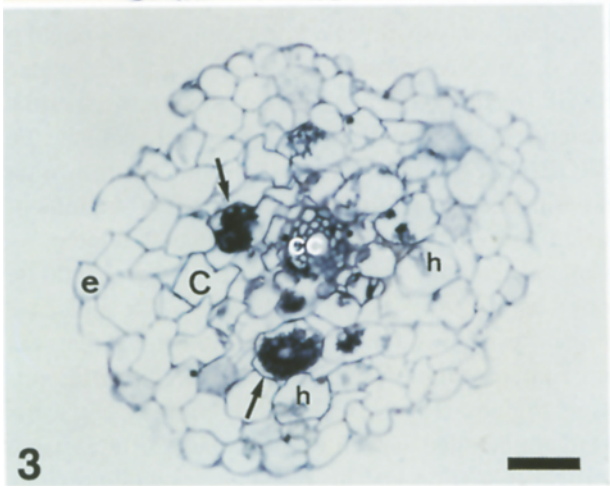
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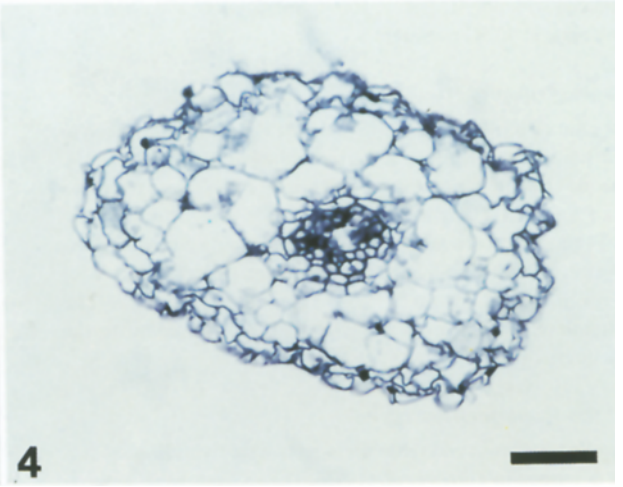
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exposure only cap cells with no HRGP mRNA were stained (Fig. 2).

In mycorrhizal roots, the fungus only colonizes differentiated cortical cells (for details, see Balestrini et al. 1994). The signal was only present in cortical cells with arbuscules (Fig. 3). No signal was present in non-colonized cells, neither in uninfected roots (Fig. 4).

Comparable results on maize root apex (result not shown) and on differentiated cells (Figs. 5–10) were also obtained when we used a ^{35}S-labelled RNA HRGP probe. The antisense-probed sections (Figs. 5 and 6) revealed the presence of the hybridization signal in cortical cells containing coiled hyphae. The signal was absent in epidermal and non-colonized cells, as well as in the central cylinder. Figures 7–9 show silver grains in some cortical cells containing arbuscules. As usual, the nucleus is centrally located and is devoid of silver grains. Control sections where the sense probe was used were not labelled (Fig. 10).

Discussion

In situ hybridization experiments demonstrate that in maize mycorrhizal roots the expression change of a HRGP gene is limited to specific cells, i.e., those containing coiled hyphae and arbuscules. The use of cold and hot probes led to the same results, but allowed us to visualize the signal at different degree. With digoxygenin, the signal was more evident, but it partly blurred the fungal hyphae. By contrast, the radioactive probe more clearly distinguished thin fungal branches and the sites where the probe was located.

Recent in situ hybridization experiments have clearly demonstrated that many transcripts involved in plant defence responses [chitinases, glucanase, phenylalinine ammonia-lyase (PAL) and chalcone synthase (CHS)] accumulate in cells containing arbuscules (Harrison and Dixon 1994, Lambais and Medhy 1995, Blee and Anderson 1996). Bonfante et al. (1996) used transgenic tobacco plants to demonstrate that the colonization by AM fungi is accompanied by changes in the transcriptional activity of the maize α-tubulin (*Tub 3*) gene and that accumulation of the corresponding transcripts is limited to the root cells containing arbuscules. Our observations provide further evidence that AM fungi also induce a very localized response of the host cells by activation of genes such as for HRGPs, which are involved in the synthesis of cell wall components. Franken and Gnädinger (1994) used RNA blot analysis from AM parsley roots to show that a transcript which codes for a HRGP is produced in much higher amounts in mycorrhizal roots than in uninfected controls. The fact that our result with RNA blot analysis did not show an evident difference in the activation of HRGP gene in maize mycorrhizal roots could be a question of limits of detection due to the dilution of the specific mRNA. The variety of maize we used shows only 40–50% of colonized cells, and not all of them display accumulation of HRGP mRNA, probably due to the fact that the gene may only be activated at specific stages. The proportion of cells accumulating HRGP mRNA, shown by the in situ hybridization, is probably too low to allow detection of differences in specific transcripts. This hypothesis is confirmed by the data obtained by Blee and Anderson (1996), who found no increase in HRGP mRNA on RNA blots of bean mycorrhizal roots with similar percentage of root colonization.

In conclusion, the present results show that *Glomus versiforme* induces an increased expression of a HRGP gene in maize roots, but only in colonized cells. We already knew that HRGP is present in the

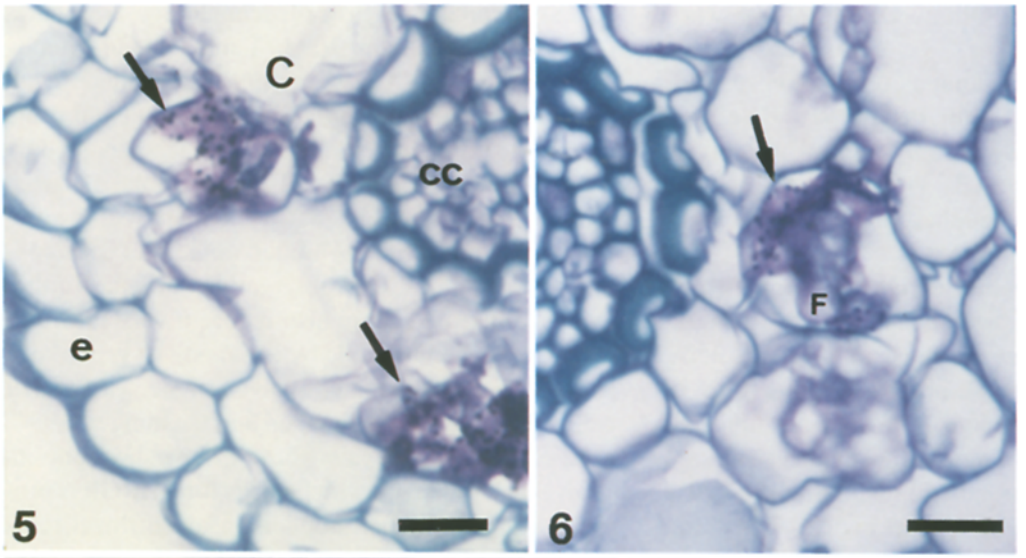
Figs. 1–4. Locations of HRGP mRNA in sections from apical (Figs. 1 and 2) and differentiated regions of maize mycorrhizal (Fig. 3) and non-mycorrhizal roots (Fig. 4) by cold in situ hybridization. *m* Meristematic cells; *c* cortical cells; *cc* central cylinder, *e* epidermal cells; *h* intracellular hyphae; *rc* root cap cells; *v* vascular tissue. Bars: Figs. 1 and 2, 200 μm; Figs. 3 and 4, 50 μm

Fig. 1. In situ hybridization of a DIG-labelled RNA antisense probe with a root apex longitudinal section. The signal is most intense in meristematic regions and on the regions of vascular differentiation (arrow). The differentiated cap cells were not labelled as well as the already differentiated cortical cells

Fig. 2. Longitudinal section of a root tip hybridized with a sense HRGP probe. This probe indicates the background level of non-specific binding in these experiments

Fig. 3. In maize mycorrhizal roots, the fungus colonizes only differentiated cortical cells. The signal is present only in the cortical cells that contained arbuscules (arrows)

Fig. 4. No signal is present in sections from uninfected roots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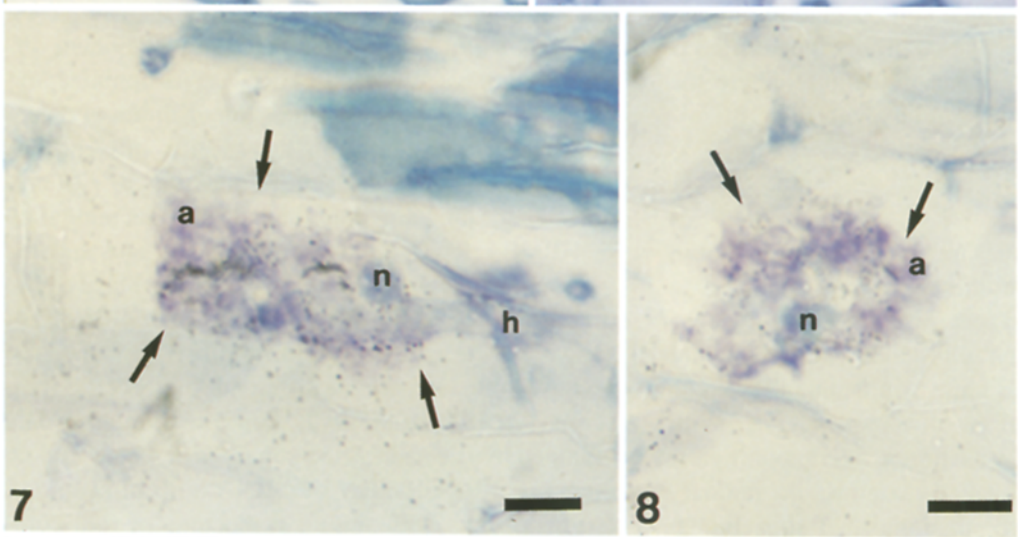
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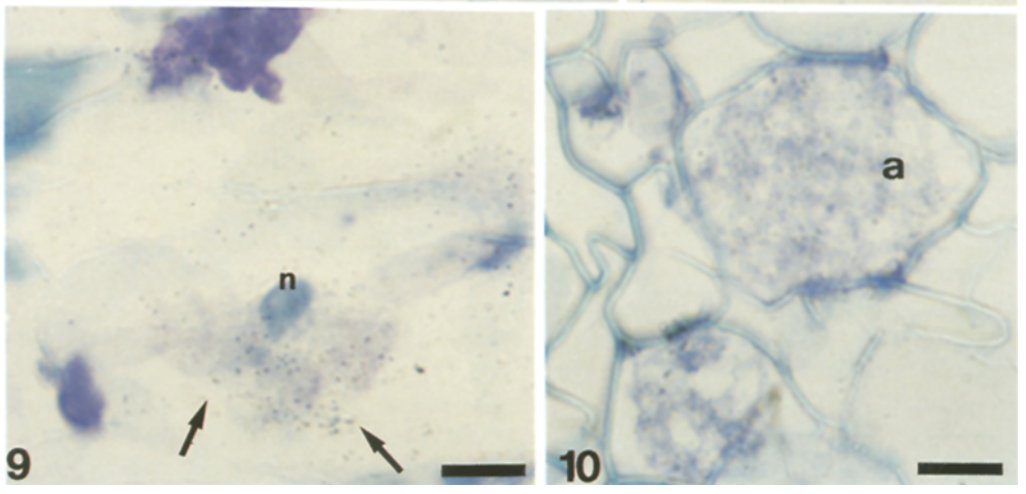
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interface between plant and fungus (Balestrini et al. 1994). On the basis of the in situ hybridization experiments, it can be considered as the result not of transport from another site, but of de novo biosynthesis after new transcription. Our data, however, cannot provide conclusive evidence whether this is an effect of a signal elicited by the fungus through (i) a pathway similar to that activated by pathogenic fungi (Benhamou et al. 1990), (ii) events related to the cell cycle (Vera et al. 1994), or (iii) specific activation of cell wall genes involved in new cell wall biogenesis. In mycorrhizal roots, HRGP gene activation may be involved in the reprogramming of infected root cells, where new cell wall material is required. The lack of a definite role for HRGP as well as its involvement in many processes means that all three hypotheses are open.

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Figs. 5–10. Location of HRGP mRNA in maize roots colonized with *G. versiforme*. Sections were hybridized with ^{35}S-labelled transcripts. The sections were stained with toluidine blue allowing structural features to be visualized by light field microscopy. Black grains on the light field micrographs indicate the locations of hybridizing ^{35}S-labelled transcripts (arrows). *a* Arbuscule; *cc* central cylinder; *c* cortical cells; *e* epidermal cells; *F* intracellular fungus; *h* arbuscule trunk; *n* host nucleus. Bars: 10 μm

Figs. 5 and **6.** Antisense-probed sections revealed the presence of the hybridization signal in cortical cells containing coiled hyphae (arrows)

Figs. 7–9. Longitudinal sections. The arrows indicate the hybridization signal in cortical cells containing arbuscules

Fig. 10. Control section where the sense probe was used is not labelled

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