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SHORT COMMUNICATION

Transcriptional activation of a maize α-tubulin gene in mycorrhizal maize and transgenic tobacco plants

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Summary

Mycorrhizae are symbioses between soil fungi and roots, with substantial modifications of the cells of both partners. Thus, host cells colonized by mycorrhizal fungi may express otherwise inactive genes. Here it is demonstrated that two arbuscular mycorrhizal (AM) fungi, *Glomus versiforme* and *Gigaspora margarita*, cause the transcriptional activation of a gene coding for α-tubulin in the colonized cells of maize and transgenic tobacco roots. Tobacco plants transformed with a construct containing the maize *Tubα 3* gene promoter fused upstream from the bacterial GUS gene showed an intense GUS activity only in meristematic regions. When colonized by *G. margarita*, GUS activity was detected in the cortical root cells containing fungal arbuscules. No GUS activity was found in root cells when transformants carrying *Tubα 1* promoter were used, nor when *Tubα 3* transformants were colonized by ericoid mycorrhizal fungi, leading to a non-specific interaction. Activation of the *Tubα 3* appears to be specific to the gene and to the appropriate interaction. Further evidence that fully differentiated host tissues re-activate the *Tubα 3* gene following colonization by AM fungi also comes from accumulation of the corresponding transcripts in maize root cells containing arbuscules.

Introduction

Arbuscular mycorrhizal (AM) fungi colonize the roots of about 80% of land plants to form symbioses which in almost all ecosystems play a central role in assimilation of nutrients from the soil (Read *et al.*, 1992). They belong to a small order within the Zygomycetes, the Glomales, whose origin has been dated to about 400 Myears ago (Simon *et al.*, 1993). It has been suggested that mycorrhizae were instrumental in colonization of the land by ancient plants (Pirozynski and Malloch, 1975).

AM fungi undergo complex morphogenesis inside the host root, with development of intracellular structures. When the spores germinate and the hyphae contact the root, they produce appressoria. These give rise to hyphae which initiate infection of root tissues by forming inter- and intracellular hyphae, coils, highly branched arbuscules and, in some cases, vesicles. Only specific tissues such as the epidermis and the cortex are colonized, whereas others, such as meristems or vascular tissues, are resistant to infection. During colonization, the host cells substantially change their organization: the nucleus moves from the periphery to a central position, amyloplasts disappear, and the central vacuole becomes fragmented (Bonfante and Perotto, 1995).

Invagination of the host membrane around the fungus creates a new compartment (the interface), considered to be the main site for nutrient exchange between the partners (Smith and Smith, 1990). Cell wall-related molecules of fungal and plant origin have been located at the interface by using affinity techniques. Results suggest that the membrane which surrounds the fungus retains the biosynthetic and secretory machinery involved in the deposition of cell wall molecules (Bonfante and Perotto, 1995). However, the molecular mechanisms controlling these events are mostly unknown. Recent reports have suggested that plant gene expression changes during the establishment of mycorrhizal symbiosis (Gianinazzi-Pearson *et al.*, 1995). However, only genes related to plant defence and characterized by a weak and transient response have so far been investigated (Franken and Gnadinger, 1994; Harrison and Dixon, 1994; Lambais and Mehdy, 1993).

The availability of transformed tobacco plants, as hosts for AM fungi, enabled us to see whether the substantial morphological changes detected in colonized cells are caused by the altered transcriptional activities of certain genes. Genes encoding for cytoskeleton elements could control organelle position as well as secretion events related to the establishment of the interface. Tobacco plants containing a construct in which the promoter of a maize α-tubulin gene was fused with the bacterial GUS gene (Montoliu *et al.*, 1989, 1990) show an intense GUS activity in meristematic tissues, including the root meristem (Rigau *et al.*, 1993). They can be used to examine the effect of mycorrhizal fungi on the transcriptional activation of one foreign α-tubulin gene. Our experiments revealed an

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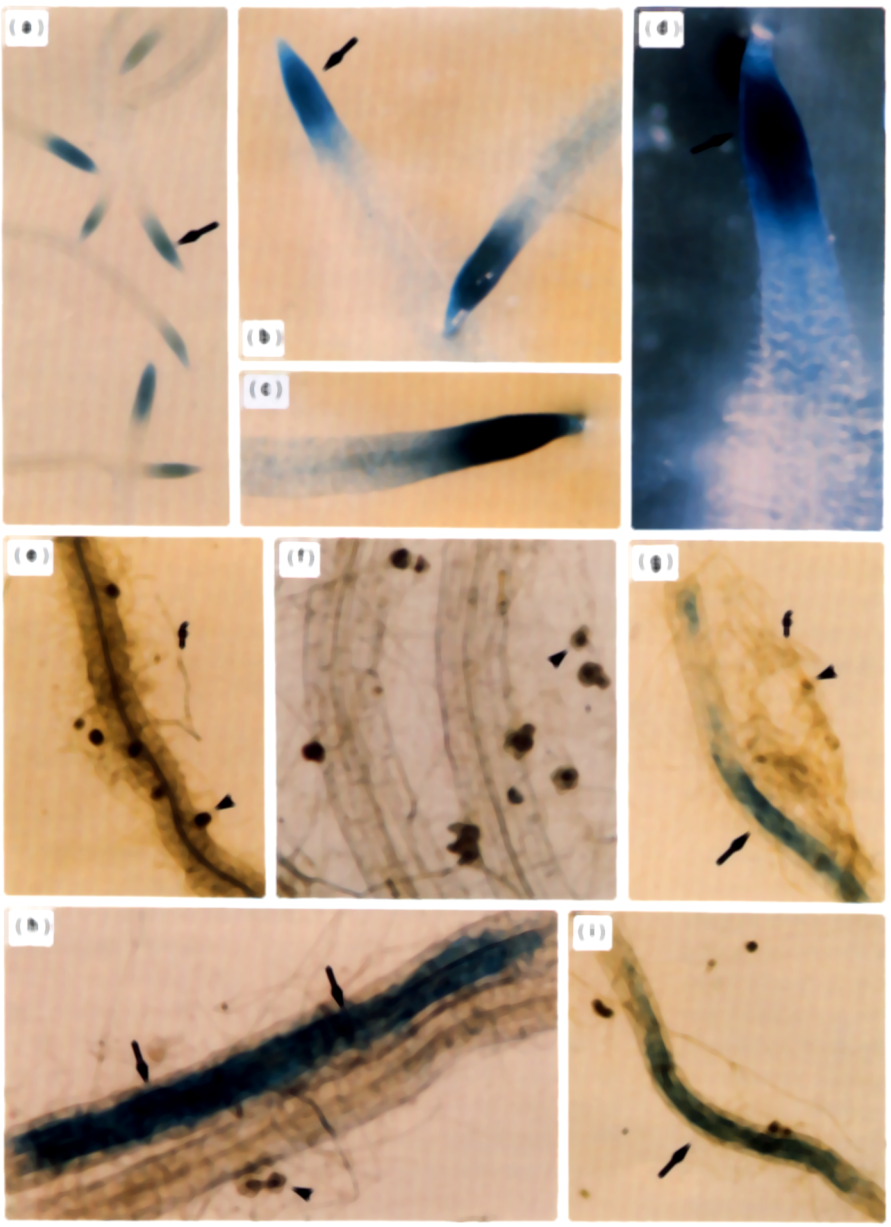

Figure 1. Histochemical localization of GUS activity in non-mycorrhizal and mycorrhizal tobacco roots.
(a–d) Non-mycorrhizal roots from *Tubα 1* (a) and *Tubα 3* (b–d) tobacco transformants: GUS activity is present in the meristem (arrows), but not in the root cap or in the differentiated regions.
(e) GUS reaction in tobacco mycorrhizal roots (wild-type). Extraradical hyphae and auxiliary cells (arrowhead) of *G. margarita* are seen around the root, while the intraradical fungal structures are hardly seen under the stereoscope microscope. The GUS reaction is negative.
(f–i) Mycorrhizal roots from the tobacco transformants: *Tubα 1* (f), *Tubα 3* with the longest construct, –1024 bp (g–h) and with the intermediate one, –633 (i). The reaction is negative with the *Tubα 1* promoter, but is positive in the other two transformants. Gus reaction is evident in the cortex (arrows), where the fungus develops. Neither extraradical hyphae nor the auxiliary cells (arrowhead) are stained. Infected root segments containing intercellular hyphae are not stained (g).
Abbreviation: f, extraradical hyphae. (a) ×8.4, (b) ×25.2, (c) ×28, (d) ×56, (e) ×22.4, (f) ×42, (g) ×19.6, (h) ×42, (i) ×19.6.

altered pattern of activation for the *Tubα 3* gene in mycorrhizal plants. Infection of differentiated cortical cells with the mycorrhizal fungus *Gigaspora margarita* induced a novel site of expression. Gene activation was only observed in the appropriate host–fungus combination. Other combinations where a necrotrophic rather than a symbiotic interaction was established did not lead to any visible changes.

Results

Promoters of maize α-tubulin genes fused upstream from the coding region of the GUS gene are active in different tissues of transgenic tobacco plants (Rigau *et al.*, 1993). Intense blue staining was found in the root tips of transgenic plants containing the promoter of either *Tubα 1* or *Tubα 3* genes (Figure 1a–d). The meristem was labelled, but not the cap or all other differentiated tissues (Figure 1d). Expression only began to be evident three weeks after germination.

To study changes in expression of tubulin genes during establishment of a mycorrhiza, transgenic tobacco plants were grown in the presence of *G. margarita*. The fungus profusely colonized tobacco roots in 2 weeks, producing the structures typical of arbuscular mycorrhizae: extraradical hyphae and auxiliary cells around the root (Figure 1e–i), inter- and intracellular hyphae and arbuscules, which were seen inside the root tissues after trypan blue staining (not shown).

After GUS staining, no differences were found between uninoculated tobacco plants (Figure 1a) and mycorrhizal transformants with the maize *Tubα 1* (Figure 1f). In contrast, transformants with the *Tubα 3* promoter showed GUS blue staining in the differentiated cortical region (Figure 1g and h). Labelling corresponded to the cortical zone where the fungus was resident in form of coils or arbuscules. Mycelium growing around the roots was not stained (Figure 1g and h), nor was the central cylinder. Along the mycorrhizal root, some root segments containing intercellular hyphae appeared GUS negative (Figure 1g). Constructs containing truncated promoters were also tested. Mycorrhizal transformants containing the intermediate construct (–633 bp) of the promoter gave a positive response in the differentiated region (Figure 1i). No expression was found in roots containing the –333 bp construct (not shown). No GUS reaction was observed in wild-type mycorrhizal tobacco plants (Figure 1e) or in the fungus growing alone (not shown).

Intraradical fungal structures in mycorrhizal *Tubα 3* transformants were more easily observed in paraffin-embedded root sections, where the lectin wheat germ agglutinin, specific for chitin, was used to reveal fungal cell walls of intercellular hyphae, large coiled hyphae and arbuscules (Figure 2a and b). When the same sections were observed under transmitted light, GUS staining was located all around the intracellular fungal hyphae (Figure 2c). No reaction was observed on similar sections from mycorrhizal transformants, where the promoter was deleted down to the 3′ terminal 333 bp (Figure 2d). Colonization of transformants for the *Tubα 3* promoter with *Glomus versiforme*, another arbuscular mycorrhizal fungus, gave identical results (not shown).

In the presence of tobacco, the ericoid fungus *Hymenoscyphus ericae* sometimes developed intracellular hyphae, which caused a localized browning, local lesions and cell death (not shown). GUS activity in transgenic plants infected by *H. ericae* was limited to the apex, as in uninfected plants, whereas no activity was detected in the differentiated regions where the fungus was growing (not shown). Another ericoid fungal strain (identified as PSIV) caused severe lesions in the tobacco seedlings, and was rapidly fatal. The results of the GUS experiments are summarized in Table 1.

To investigate whether the presence of the mycorrhizal fungus would also activate *Tubα 3* gene in maize, *in situ* hybridization experiments were carried out on maize roots infected with *Glomus versiforme*. A detailed description of the mycorrhizal infection in maize is given by Balestrini *et al.* (1994). In mycorrhizal roots, the hybridization signal was seen in the infected cells around coiled or arbuscular hyphae (Figure 3b and c), whereas control sections where the sense probe was used were not labelled (Figure 3a). In non-mycorrhizal maize roots, *Tubα 3* transcripts were only found in the apical meristem, in agreement with the gene expression pattern observed by RNA blot experiments (Montoliu *et al.*, 1990).

Discussion

Discovering the mechanisms responsible for the metabolic and physiological changes well described in mycorrhizal roots remains a challenge. This paper offers evidence that a gene coding for an α-tubulin, a cytoskeletal protein, changes its expression during the establishment of symbiosis with arbuscular mycorrhizal fungi in both maize and transgenic tobacco.

Expression of tubulin genes in plant–microbe interactions

Tubulins are components of microtubules and play a crucial role in plant form and development (Lloyd, 1991) through their control of cytoplasm organization, cell division, orientation of cell-wall components, and subsequent elongation and cell growth. Since the plant cytoskeleton rapidly responds to extracellular stimuli, it may also play important roles in plant–microbe interactions, such as in legume–*Rhizobium* symbiosis (Bakhuizen, 1988; Rae *et al.*, 1992). When differentiated root cortical cells become mycorrhizal, they are extensively reorganized (Bonfante and Perotto, 1995). Morphological changes involve fragmentation of the central vacuole, movement of the nucleus and other organelles towards the fungal branches, and *de novo* formation of a subcellular interface compartment where plant cell surface molecules are newly laid down (Bonfante and Perotto, 1995). During these changes, the cytoskeleton is reorganized, and immunofluorescence reveals a very different array of microtubules (Genre and Bonfante, unpublished results).

The present results show that the reorganization of root cortical cells upon colonization by arbuscular mycorrhizal fungi is accompanied by changes in the transcriptional activity of the *Tubα 3* gene, and indicate that new biosynthesis of tubulin components is required. GUS experiments on mycorrhizal transgenic tobacco plants have shown that the cortical cells colonized by the fungus are a specific and new *Tubα 3* expression site. *In situ* hybridization experiments on maize mycorrhizal roots have shown comparable changes in the homologous system, since they reveal the presence of specific transcripts in differentiated cortical cells containing fungal arbuscules.

Tubα 1 *and* 3 *are differently involved in the re-programming of mycorrhizal cells*

Tubulins are encoded by multigene families. They have been studied in a few plant species including maize (Montoliu *et al.*, 1992; Rigau *et al.*, 1993; Silflow *et al.*,

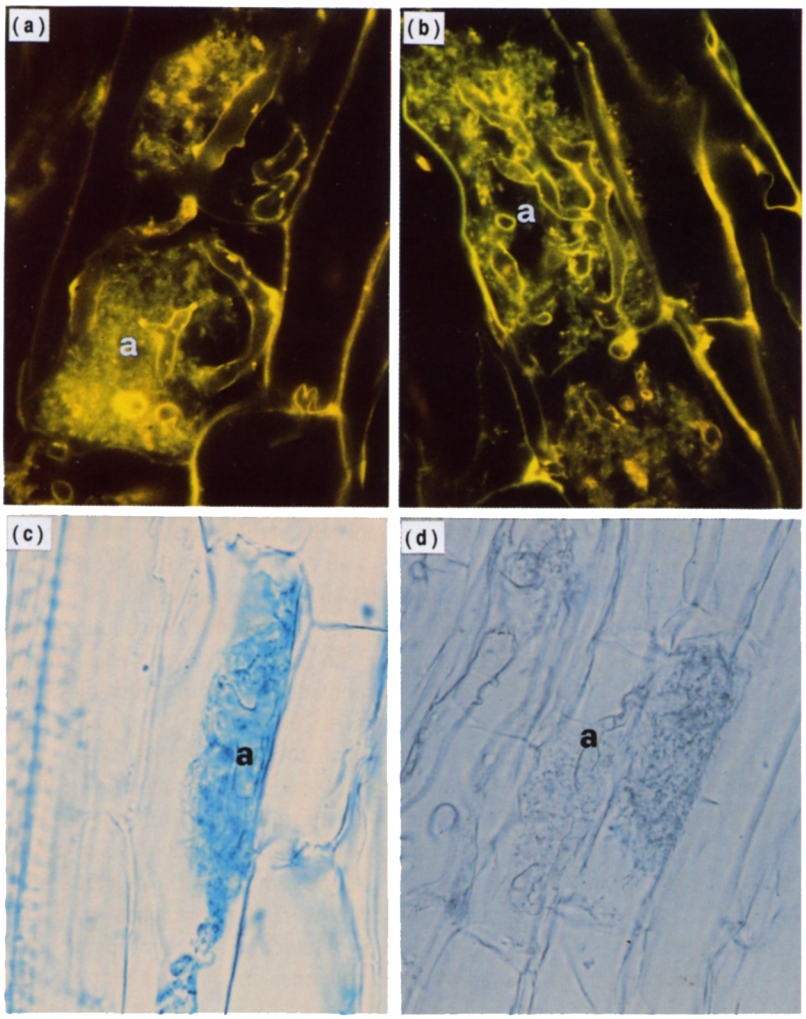

Figure 2. Paraffin sections of mycorrhizal roots from tobacco transformants, colonized by *G. margarita*.
Histochemical localization of GUS activity of the transcriptional fusion with the −1024 (a–c) and the −333 construct (d) of the *Tubα 3* gene promoter.
(a and b) Sections are treated with the WGA/FITC complex and seen under fluorescent light. Large intracellular hyphae, and branched arbuscules are seen in the cortical cells.
(c) A serial section is observed under the transmitted light. GUS deposits are seen in the host cytoplasm of a cortical cell harbouring a fungal arbuscule. They tightly surround the fungal walls. There is no staining in the non-infected cells.
(d) No GUS reaction is seen in a similar section from the transformant carrying the larger deletion (d).
Abbreviation: a, arbuscules. (a and b) ×469, (c and d) ×335.

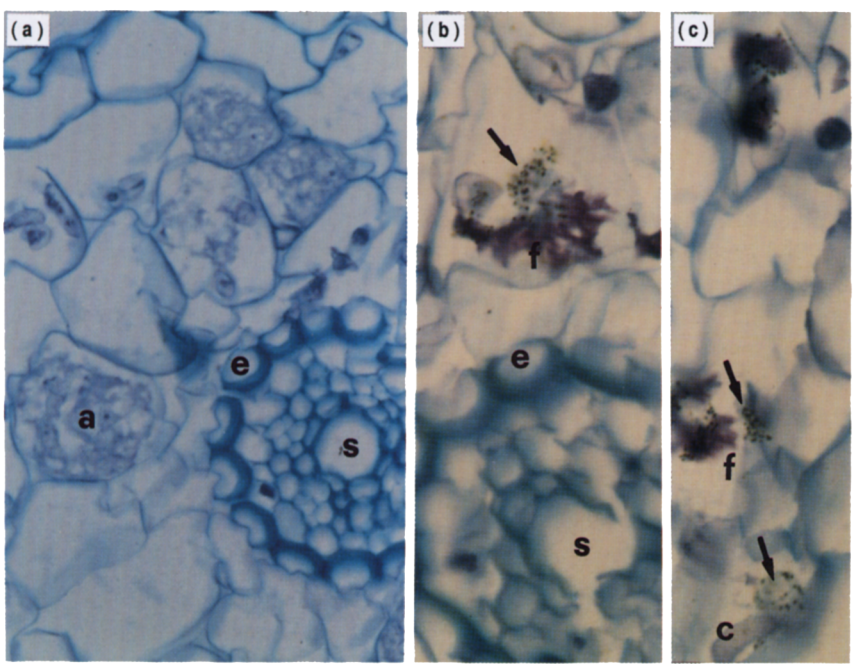

Figure 3. *In situ* hybridization in maize mycorrhizal roots, using a ^{35}S-labelled riboprobe from a 230 bp fragment of the 3′ non-coding region of the *Tubα 3* gene.
(a) A root section harbouring arbuscules of *Glomus versiforme* and treated with the sense riboprobe, does not show any silver granules. (b and c). As (a), but with the antisense riboprobe. Silver granules are present around the collapsed fungal arbuscular branches (arrows) and around coiled hyphae. No labelling on the uninfected cells, on the endodermis or in the stele.
Abbreviations: a, arbuscules; c, coiled hyphae; f, intraradical hyphae; e, endodermis; s, stele.
(a) ×201, (b and c) ×301.

Table 1. Results from GUS reaction experiments performed on transformant *Tubα 3* tobacco seedlings in the presence of endomycorrhizal fungi

Fungal species	Mycorrhizal type	Roots	
		Meristems	Cortical cells
Gigaspora margarita Becher & Hall	Arbuscular	+	+
Glomus versiforme (Karst.) Berch	Arbuscular	+	+
Hymenoscyphus ericae (Read) Korf & Kernan	Ericoid	+	–
Sterile mycelium PS IV	Ericoid	+	–[a]

[a]With necrotic areas.

1987; Villemur *et al.*, 1992), *Arabidopsis thaliana* (Ludwig *et al.*, 1988) and carrot (Hussey *et al.*, 1988). Differential expression of multiple α- and β-tubulin genes has been revealed during tissue development and in response to external stimuli (Hussey *et al.*, 1991). A 'multi-tubulin hypothesis' (Fulton and Simpson, 1976), according to which every isotype has a different function, has been proposed. Our results indicate that this may also be the case in plants, as genes belonging to different subfamilies of α-tubulins are differentially expressed during the establishment of arbuscular mycorrhiza. *Tubα 3* is activated in the colonized root cells, whereas *Tubα 1* is not. *Tubα 1* is certainly involved in nuclear divisions (Montoliu *et al.*, 1989; Rigau *et al.*, 1993), and its lack of expression during the establishment of arbuscular mycorrhizae is in accord with the observation that nuclei change their position, but do not undergo mitosis (Balestrini *et al.*, 1992). The *Tubα 3* product may be more involved in the cell changes occurring during mycorrhizal infection, including organization of the interface compartment, which requires co-ordinate secretion of matrix material, plant plasma membrane and cell-wall material (Bonfante and Perotto, 1995). Lund *et al.* (1995) showed that α-tubulin genes have different roles in maize endosperm, where nuclear divisions and cell-wall formation are crucial events.

Arbuscular mycorrhizal fungi cause specific activation of the crucial α-tubulin gene in a way analogous to that demonstrated during the development of nematode feeding structures in *Arabidopsis*. Nematodes can cause reprogramming of root cells, and consequent activation or downregulation of specific genes (Goddijn *et al.*, 1993). As has been suggested for root–nematode interactions (Opperman *et al.*, 1994), during the evolution of mycorrhizal symbiosis arbuscular fungi may have recruited plant genes to be expressed in specific developmental and temporal patterns. It will now be of interest to determine whether gene activation involves direct interaction of fungal signals with the α-tubulin promoter, or indirect interaction through a developmental cascade.

Experimental procedures

Plant transformation and regeneration

All studies were done with *Nicotiana tabacum* L. cv. Petit Havana SR1 or maize W64 pure inbred line grown in the greenhouse. Tobacco plants were transformed using *Agrobacterium tumefaciens* and the GUS reporter gene, and constructs with the *Tubα 1* and *Tubα 3* promoters were made as described by Rigau *et al.* (1993). Constructs corresponding to gene *Tubα 3* ended at position +53 from the transcription start site. The longest construct was 1076 bp long, beginning at position –1024, the intermediate construct began at –633, and the shortest one at position –333 (Montoliu *et al.*, 1990).

Assays for mycorrhizal infection

Surface-sterilized seeds (10 min in 1% sodium hypochlorite) of transgenic and non-transgenic tobacco plants were germinated in petri dishes and brought into contact with the fungus 10 days after germination, using the millipore sandwich method (Giovannetti *et al.*, 1993). Briefly, the seedlings were placed between two millipore membranes (Millipore, 0.45 mm diameter pores), one containing 10–15 pregerminated spores of *Gigaspora margarita* Becher & Hall. The sandwich was then transplanted into pots of sterile acid-washed quartz sand (2–5 mm). Controls were set up without the spores.

The seedlings were grown under a 16 h photoperiod at 60% relative humidity, and watered with a nutrient solution. After 20–25 days, the sandwiches were opened and fungal development was assessed under a microscope. One part of the root systems was stained with 0.05% trypan blue in lactic acid to reveal the mycorrhizal structures, while the other part was used for GUS analysis.

In parallel experiments, another arbuscular mycorrhizal fungus *Glomus versiforme* (Karst.) Berch was used. Ungerminated spores were used to initiate the infection. To check the pattern of α-tubulin expression in tobacco roots in the presence of a non specific mycorrhizal fungus, transgenic tobacco seedlings were put in contact with growing cultures of two endomycorrhizal fungi, *Hymenoscyphus ericae* (Read) Korf & Kernan and the sterile strain PS IV. These fungi establish symbioses with ericaceous plants, whereas tobacco is a non-host, like clover and maize (Perotto *et al.*, 1995, for a review on the ericoid systems).

Mycorrhizal plants of maize were obtained as described in Balestrini *et al.* (1994).

Histochemical analysis

GUS assays were carried out according to Jefferson *et al.* (1987). Mycorrhizal and non-mycorrhizal roots from about 120 seedlings of the same age, and germinated spores of *G. margarita* were stained directly for GUS activity at 37°C for 14–16 h. Tissues were assessed under a Leitz stereoscope. Alternatively, other GUS-stained samples were embedded in paraffin (melting point 52°–54°C) and 8 μm sections were cut with a standard rotary microtome (Altamura *et al.*, 1991). To better reveal the fungal structures, some sections were treated with wheat germ agglutinin (WGA), bound to FITC (Bonfante *et al.*, 1990). In this case, the sections were viewed under a Bio-Rad View Scan Microscope using an argon laser.

Mycorrhizal and non-mycorrhizal maize roots were embedded in paraffin according to Pancoro and Hughes (1992). *In situ* hybridization of paraffin sections with the *Tubα 3* probe was carried out using riboprobes from a 230 bp fragment of the 3′ non-coding region of the *Tubα 3* clone (Montoliu *et al.*, 1990) cloned in pBluescript SK (Stratagene) and radioactive labelling by using 35S-CTP (Amersham). Both sense and antisense probes were used for hybridization experiments. The procedure was essentially that described by Langdale (1993).

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