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Differential location of α -expansin proteins during the accommodation of root cells to an arbuscular mycorrhizal fungus

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Abstract α -Expansins are extracellular proteins that increase plant cell-wall extensibility. We analysed their pattern of expression in cucumber roots in the presence and in the absence of the mycorrhizal fungus, *Glomus versiforme*. The distribution of α -expansins was investigated by use of two polyclonal antibodies (anti-EXPA1 and anti-EXPA2, prepared against two different cucumber α -expansins) in immunoblotting, immunofluorescence, and immunogold experiments. Immunoblot results indicate the presence of a 30-kDa band specific for mycorrhizal roots. The two antibodies identify antigens with a different distribution in mycorrhizal roots: anti-EXPA1 labels the interface zone, but the plant cell walls only weakly. By contrast, the anti-EXPA2 labels only the plant cell walls. In order to understand the potential role of α -expansins during the accommodation of the fungus inside root cells, we prepared semi-thin sections to measure the size of cortical cells and the thickness of cortical cell walls in mycorrhizal and non-mycorrhizal root. Mycorrhizal cortical cells were significantly larger than non-mycorrhizal cells and had thicker cell walls. In double-labelling experiments with cellobiohydrolase–gold complex, we observed that cellulose was co-localized with α -expansins. Taken together, the results demonstrate that α -expansins are more abundant in the cucumber cell walls upon mycorrhizal infection; we propose that these wall-loosening proteins are directly involved in the accommodation of the fungus by infected cortical cells.

Keywords Arbuscular mycorrhizae · Expansin · Cell expansion · Cellulose · Cell wall

Abbreviations AM: Arbuscular mycorrhiza(l) · EXPA: α -Expansin · EXPB: β -Expansin · CBH: Cellobiohydrolase · XET: Xyloglucan endotransglycosylase

Introduction

Plant cell walls form a unique extracellular matrix that controls growth and development in the absence of cell migration (Reiter 1998). They also protect the protoplast from a range of environmental stresses, and take part in various developmental processes. Current models suggest that the plant cell wall consists of three interwoven domains: one network of cellulose and hemicellulose, another of heterogeneous pectins, and a third of structural proteins (Carpita and Gibeaut 1993). The cell wall also contains a large number of enzymes and other defence-related proteins such as polygalacturonase inhibiting proteins (PGIPs). About a decade ago, a new class of plant cell-wall proteins, called expansins, were identified and characterized as key cell-wall-loosening agents (McQueen-Mason et al. 1992). The cell-wall capacity for extension is an essential property for plant growth and morphogenesis. Biophysical analyses of expanding cells have shown that, in general, cells grow as a result of the loosening of the wall rather than an increase in turgor (Cosgrove 1993). Expansins are a group of extracellular proteins with characteristic wall-loosening activity and are involved in plant cell growth, as well as other developmental processes that require cell-wall loosening (Cosgrove 1999, 2001). They also increase plant cell-wall extensibility in vitro and directly modify the mechanical properties of plant cell walls. Two families of expansins have been shown to have wall-loosening activity, and these have been named α - and β -expansins (EXPA and EXPB; Cosgrove et al. 1997).

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Reduction of α -expansin gene expression by antisense methods results in growth inhibition (Cho and Cosgrove 2000), whereas ectopic over-expression of α -expansin on shoot apical meristems promotes leaf growth and can modify the pattern of leaf initiation (Pien et al. 2001).

Since they were first isolated from cucumber hypocotyls, expansin genes and proteins have been identified in many plant species, being restricted largely to the plant kingdom (Cosgrove 2000). Expansins are encoded by multigene families (EXPA and EXPB) and each gene is often expressed in a highly specific location and cell type. Phylogenetic analysis of expansin sequences found in monocots, pine, fern, and moss indicates that plant expansins arose and began diversifying very early during colonization of land by plants (Li et al. 2002). Distantly related "expansin-like" sequences were also identified in the social amoeba, *Dictyostelium discoideum*, suggesting that these cell-wall proteins have a very deep evolutionary origin or, alternatively, that their genes have been involved in lateral gene transfer. A potential example of lateral gene transfer may be found in the cyst-forming nematode that synthesizes and excretes a protein with expansin-like activity (Qin et al. 2004). Although the divergent structure and sequence of this protein shows that it is not a canonical expansin according to the recent definition of this gene superfamily (Kende et al. 2004), the nematode protein is nevertheless a case of an expansin-like protein being adapted for a novel biological function (cyst formation). Because expansins are involved in wall loosening, they are detected in many crucial events in addition to cell enlargement: e.g. pollen tube invasion of the stigma (in grasses), wall loosening, and disassembly during fruit growth and ripening, abscission, and other cell-separation events (Cosgrove 1999, 2000; Rose and Bennet 1999). Expansin genes are differentially regulated by environmental and hormonal signals, and hormonal regulatory elements have been found in their promoter regions (Lee et al. 2001). The tomato expansins *LeEXPA1* and *LeEXPA2*, the deep water rice expansin *OsExp4*, and the soybean (*Glycine max*) β -expansin Cim1 accumulate in response to ethylene, auxin, gibberellin, and cytokinin, respectively (Downes et al. 2001; Catala et al. 2000).

In contrast, the regulation of expansins to biotic factors has rarely been investigated. Among the microbes which have an impact on plants, nitrogen-fixing bacteria and arbuscular mycorrhizal (AM) fungi are very important. The last-mentioned are members of the phylum Glomeromycota, class Glomeromycetes (Schüßler et al. 2001) and colonize the roots of about 80% of land plants to form symbioses, which play crucial roles in plant nutrition and health (Harrison 1999). The notion that cell walls are involved in plant-defence responses and are deeply involved in the molecular dialogue between plant and microbial cells in heterologous communication has encouraged investigation of the role of cell walls in mycorrhizae (Bonfante 2001).

Each step of AM colonization is under the control of a plant genetic program from the perception of the microbial signalling molecules to the activation of symbiotic plant responses, including symbiosis-related gene activation and the induction of an intracellular "accommodation" programme (Parniske 2000; Kistner and Parniske 2002). This term refers to the substantial changes which take place in the structure of host cells following the fungal colonization (Bonfante 2001). In particular, a new apoplastic compartment (the interface) is created by the invagination of the host membrane around the fungus, where molecules common to the plant's primary wall are laid down. β -1,4-glucans, non-esterified homogalacturonans, xyloglucans, proteins rich in hydroxyproline (HRGPs), and arabinogalactan proteins (AGPs) have been located at the interface in many different plant/AM fungus combinations (reviewed by Bonfante 2001). Genes encoding a putative AGP and an HRGP are induced in mycorrhizal roots of *Medicago truncatula* and maize, respectively, and the transcripts are localized specifically in the cells containing arbuscules (vanBuuren et al. 1999; Balestrini et al. 1997). Deposition of cell-wall material often involves the combined activities of both polysaccharide synthases and lytic enzymes. Two xyloglucan endo-transglycosylase (XET) genes were isolated from *M. truncatula* (Maldonado-Mendoza and Harrison 1998), one being expressed only in mycorrhizal roots. The authors suggested that the gene product may be involved either in facilitating hyphae penetration by allowing localized cell-wall loosening or in modifying the structure of xyloglucans in the interface compartment.

Unlike the situation found for N_2 -fixing nodules, where an expansin gene has been demonstrated to be upregulated (Giordano and Hirsch 2004), the expression and localization of expansins have not yet been investigated in a plant/fungus association. The aim of this work was to test whether the presence of an AM fungus has an impact on the localization of expansins in cucumber roots. As a first step, expansins were localized by using polyclonal antibodies in immunolocalization experiments. As a second step, and in order to understand the potential role of expansins during the accommodation process of the intracellular fungus inside host cells, cell size was measured and a co-localization between expansins and cellulose was revealed.

Materials and methods

Biological materials

Surface-sterilized cucumber seeds (*Cucumis sativus* L.) cv Marketmore (Sementi Dotto, Italy) were germinated in Petri dishes. Five-day-old seedlings were transferred to pots filled with sterile quartz sand, and mycorrhizal plants were obtained by inoculating seedlings with a spore suspension obtained from *Glomus versiforme* (Karst) Berch (BEG number 47) fruitbodies. All plants

were watered three times a week with a Long Ashton solution (Balestrini et al. 1996) and maintained in a growth chamber at 22°C for about 2 months.

Protein extraction and immunoblotting

Hypocotyls, mycorrhizal, and non-mycorrhizal roots of *C. sativus* L. were homogenized in liquid nitrogen and wall proteins were extracted as described by McQueen-Mason et al. (1992). The protein extracts were then collected directly in Laemmli sample buffer, boiled, and centrifuged at 14,000 rpm for 10 min. Samples were separated by SDS-PAGE using 12% polyacrylamide gels. For immunoblotting, proteins were transferred electrophoretically to the nitrocellulose membrane and immunolabelled as described in Perotto et al. (1997) with the polyclonal antibodies anti-EXPA1 and anti-EXPA2, produced against cucumber expansins EXPA1 and EXPA2, respectively (Li et al. 1993). The two antibody preparations have selectivity for different EXPA isoforms, and were used in this study to distinguish different expansin proteins. Experiments were repeated two times.

Immunofluorescence

Immunofluorescence experiments were performed on mycorrhizal and non-mycorrhizal cucumber roots. Semi-thin sections obtained from the embedded samples (see next paragraph) were used. Sections from both the samples were incubated overnight at 4°C with the polyclonal antibodies anti-EXPA1 and anti-EXPA2 (dilution 1:500) in phosphate buffer containing 1% (w/v) BSA. Sections were washed three times for 15 min in phosphate buffer, saturated for 30 min with 1% (w/v) BSA in phosphate buffer, and incubated at room temperature in the dark for 3 h with a goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (dilution 1:80). The sections were washed as before, mounted, and observed using a confocal laser scanning microscope (Optiphot-2 View Scan DVC-250, Nikon) at 494 nm. Labelling specificity was determined by replacing the primary antibody with the buffer.

Transmission electron microscopy (TEM) and immunolabelling

For TEM, differentiated segments from uninfected and mycorrhizal cucumber roots were fixed in 2.5% (v/v) glutaraldehyde in 10 mM Na-phosphate buffer (pH 7.2) overnight at 4°C. After washing in the same buffer, the samples were treated according to two protocols. In the first procedure, they were post-fixed in 1% (w/v) osmium tetroxide in water for 1 h, washed three times with water, and dehydrated in an ethanol series [30, 50, 70, 90, and 100% (v/v); 15 min each step] at room temperature. The samples were infiltrated in 2:1 (v/v) ethanol/

LR White (Polysciences, Warrington, PA, USA) for 1 h, 1:2 (v/v) ethanol/LR White resin for 2 h, 100% LR White overnight at 4°C, and embedded in LR White resin, according to Balestrini et al. (1996). In the second procedure, they were dehydrated and embedded in LR White following a low-temperature protocol (without osmium). Semi-thin sections (1 µm) were stained with 1% (w/v) toluidine blue for morphological observations. Thin sections were post-stained with periodic acid-thiocarbohydrazide-silver proteinate test (PATAg test) for visualization of polysaccharides (Bonfante et al. 1990) or treated according to the immunolocalization protocol followed. Immunogold labelling with the polyclonal antibodies anti-EXPA1 or anti-EXPA2 (dilution 1:500–1:1000) were performed on thin sections as described by Balestrini et al. (1996) and observed with a Philips CM10 transmission electron microscope. Labelling specificity was determined by replacing the primary antibody with the buffer. The immunogold experiments were replicated about six times for each treatment.

Colloidal gold conjugate

The enzyme cellobiohydrolase (CBH I, EC 3.2.1.91, from *Trichoderma reesei*) was used to ultrastructurally reveal β -1,4-glucans. Gold complex and cytochemical labelling were performed as described in Bonfante et al. (1990). Briefly, the pH of 10 ml of 20-nm colloidal gold solution was adjusted to 4.5. A 74-µl aliquot of a stock solution of CBH I (2 mg/ml) was added. After 5 min, 500 µl of 1% polyethylene glycol (PEG) was added to the enzyme-gold mixture. The latter was centrifuged at 14,000 rpm using a Beckman rotor Ti (Beckman L5-50 Ultracentrifuge) for 1 h at 4°C. The mobile pellet was collected in 5 ml of 0.05 M citrate-phosphate buffer (CPB) pH 4.8, to which 0.02% PEG was added. The complex was stored at 4°C.

Thin sections, handled as floating sections, were treated with 0.05 M CPB for 10 min, and then incubated for 40 min with a 1:20 dilution of the enzyme-gold complex with the incubation buffer. Sections were washed in the incubation buffer for 10 min, rinsed twice in distilled water and then post-stained with uranyl and lead salts. Control sections were treated with an enzyme-gold complex solution to which carboxymethyl-cellulose had been added, at 1 mg/ml final concentration, at least 1 h before use (Bonfante et al. 1990). In double-labelling experiments, β -1,4-glucans were detected by using the same protocol following treatment with the anti-EXPA2 antibody.

Morphometry

Semi-thin sections obtained from embedded samples were used. The longest axis of cortical cells from mycorrhizal and non-mycorrhizal roots was measured

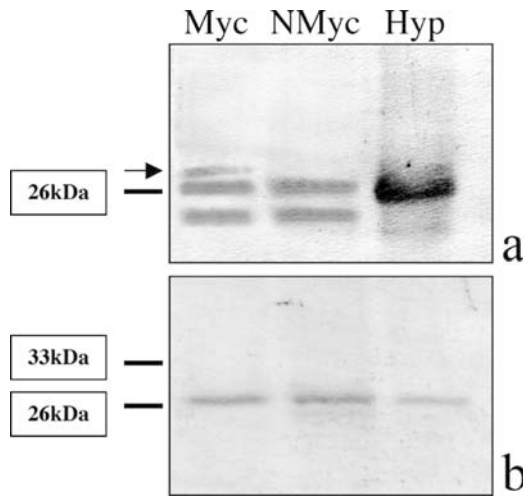


Fig. 1 Immunodetection of expansins in cucumber roots, cucumber/*Glomus versiforme* mycorrhizae and hypocotyls by using anti-EXPA1 (a) and anti-EXPA2 (b) polyclonal antibodies. Arrow indicates the additional band found in mycorrhizal roots. The migration positions of molecular-mass markers are indicated. *Myc* Mycorrhizal roots, *Nmyc* non-mycorrhizal roots, *Hyp* hypocotyls

with a micrometer under a light microscope in at least 200 medially sectioned cells. Width and length of cortical cells were determined in 100 longitudinally sectioned cells. Statistics were obtained by analysis of variance ($P < 0.05$ significant and $P < 0.01$ highly significant) and standard deviation (SD) was calculated.

The thickness of cortical cell walls from mycorrhizal and non-mycorrhizal roots was also determined on prints from electron micrographs at the same magnification (after PATAg test). Statistics were obtained by analysis of variance ($P < 0.05$ significant and $P < 0.01$ highly significant) and SD was calculated.

Results

To identify the location and relative abundance of different α -expansin isoforms in mycorrhizal roots, poly-

clonal antibodies (anti-EXPA1 and anti-EXPA2), raised against two expansins (EXPA1 and EXPA2) of cucumber hypocotyls (Li et al. 1993), were used in immunoblotting and immunolocalization studies on non-mycorrhizal and mycorrhizal root samples.

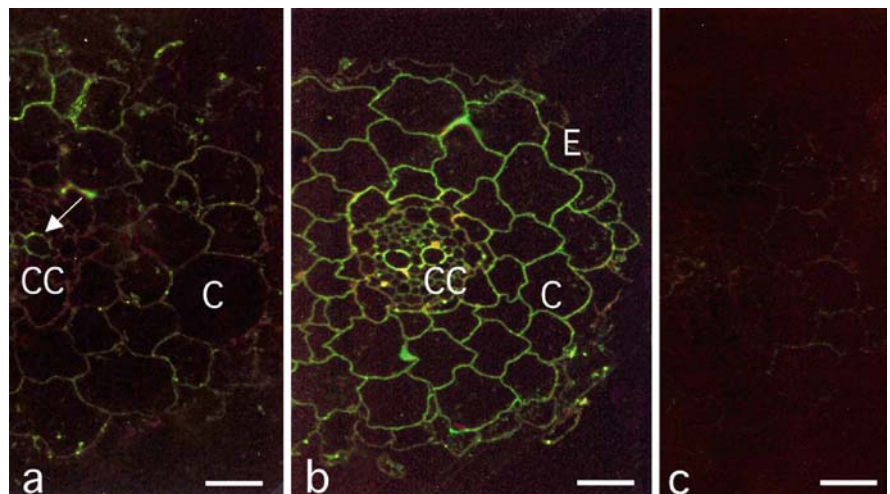
Detection of expansins in cucumber/*G. versiforme* mycorrhizae

With anti-EXPA1, two α -expansin bands were detected by immunoblot both in non-mycorrhizal and mycorrhizal root samples (Fig. 1a, lanes 1 and 2). Their apparent size was similar to that of hypocotyl α -expansins (Fig. 1a, lane 3). In addition, a band of about 30 kDa specific for mycorrhizal roots was revealed (Fig. 1a, lane 1). By contrast, no difference was revealed by using the anti-EXPA2, where a single band was evident in all samples (Fig. 1b). In both membranes, a protein sample extracted from 4-day-old cucumber hypocotyls was loaded as a positive control. However, the specific band of about 30 kDa was not further characterized since the substantial amounts of protein required for the purification of expansins were not attainable in our experimental system (Rochange and McQueen-Mason 2000).

Localization of antigens recognized by anti-EXPA1 and anti-EXPA2 antibodies

The EXPA1 and EXPA2 antibodies were used in immunofluorescence and immunogold experiments in order to detect the distribution of EXPA proteins in the differentiated regions of non-mycorrhizal and mycorrhizal roots. In immunofluorescence, a green fluorescent signal was detected on the plant cell walls of the different non-mycorrhizal root tissues labelled with anti-EXPA2 (Fig. 2b), while a weak green fluorescence was revealed by anti-EXPA1 (Fig. 2a). In the presence of the AM fungus, intense labelling by anti-EXPA1 was found

Fig. 2 Detection of expansins in cucumber roots after immunofluorescence labelling on semi-thin sections. **a** In control roots, a weak labelling is detected associated to the cell wall by using anti-EXPA1. Arrow indicates the labelling on the cell walls in vascular tissue. **b** After treatment with the anti-EXPA2, a more intense green fluorescent signal is present on the plant cell walls of the root tissues. **c** No signal is found in control sections where the primary antibodies are omitted. C Cortical cell, CC central cylinder, E epidermal cell. Bars 20 μ m



mostly around the fungal arbuscules, i.e. the intracellular structures, which colonize the cortical root cells (Fig. 3a, b). By contrast, anti-EXPA2 led to a regular labelling on the plant cell walls but not around the intracellular fungus (Fig. 3c). No signal was revealed in the control section where the primary antibodies were omitted (Fig. 3d).

Under electron microscope level, anti-EXPA1 led to an intense labelling on the cell walls only in vascular tissue (Fig. 4b), but not on the epidermal (Fig. 4a) or cortical (not shown) cell walls of non-mycorrhizal roots, where only a few gold granules were found. After the treatment with anti-EXPA2, a consistent labelling was present on the cell walls of all tissues: epidermis, cortex, and central cylinder (Fig. 4c–e), confirming the picture observed by fluorescence microscopy. In the presence of the AM fungus, EXPA1 antigens were clearly detected in the interface zone (Fig. 5b), limited by the host membrane and the fungal wall. They were not present on the fungal cell wall, while a few gold granules were associated to the wall of cortical cells (Fig. 5a). After

treatment with anti-EXPA2 on the same material, gold granules were observed on the cell walls of the root tissues. The distribution of the labelling was comparable with that observed in the non-mycorrhizal roots. In this case, however, gold granules seemed to be particularly abundant on the cell walls surrounding the intercellular hyphae (Fig. 5c). No labelling was found in the interface zone (Fig. 5d).

Morphometric analysis

In order to verify whether the responses set up by the plant cell following the fungal penetration were related to wall loosening and cell expansion, the size of cortical cells was measured in non-mycorrhizal and mycorrhizal roots. The measurements were limited to cells containing the intracellular fungus. The cortical cells containing the AM fungus were significantly larger than the corresponding cortical cells from non-mycorrhizal roots (Table 1). The results also showed that the presence of

Fig. 3 Detection of expansins in mycorrhizal cucumber roots after immunofluorescence labelling on semi-thin sections. Treatment with anti-EXPA1 (a, b) or anti-EXPA2 (c, inset). **a** After treatment with anti-EXPA1, an intense signal is present mostly around the fungal intracellular structures (arrows). Bar 9 μm . **b** Higher magnification of a cortical cell containing an arbuscule: a fluorescent signal is also associated to the plant cell walls. Bar 7 μm . **c** A regular labelling on the plant cell walls is detected with the anti-EXPA2, similar to that shown in the control roots (Fig. 2b). *Inset* Magnification of cortical cells containing arbuscules: no specific signal is detected around the intracellular fungus, while a green fluorescent signal is present on the plant cell walls. Bar 9 μm for (c) and 8 μm for the *inset*. **d** No signal is found in control sections where the primary antibodies are omitted. Bar 17 μm . **e** Semi-thin section illustrates the colonization in the cucumber roots. Arrows point to intercellular hyphae. Bar 15 μm . A Arbuscule, C cortical cell, CC central cylinder, E epidermal cell

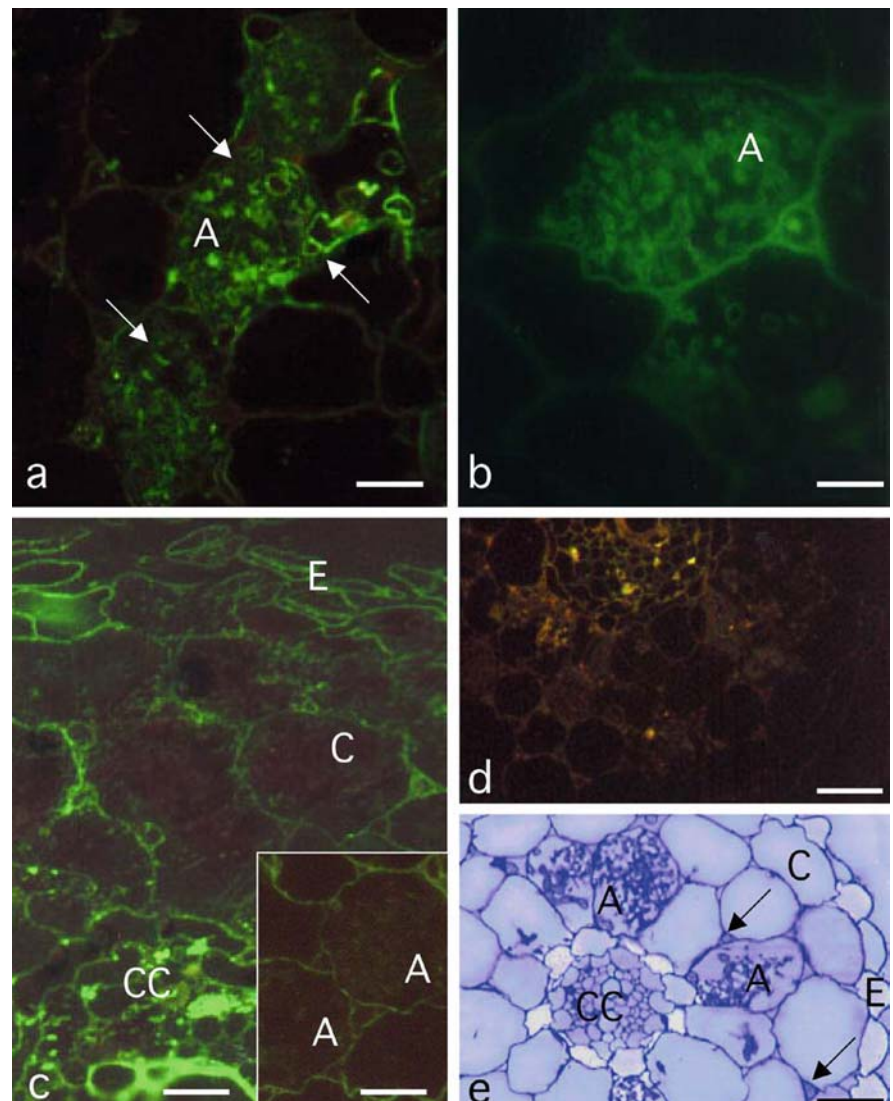
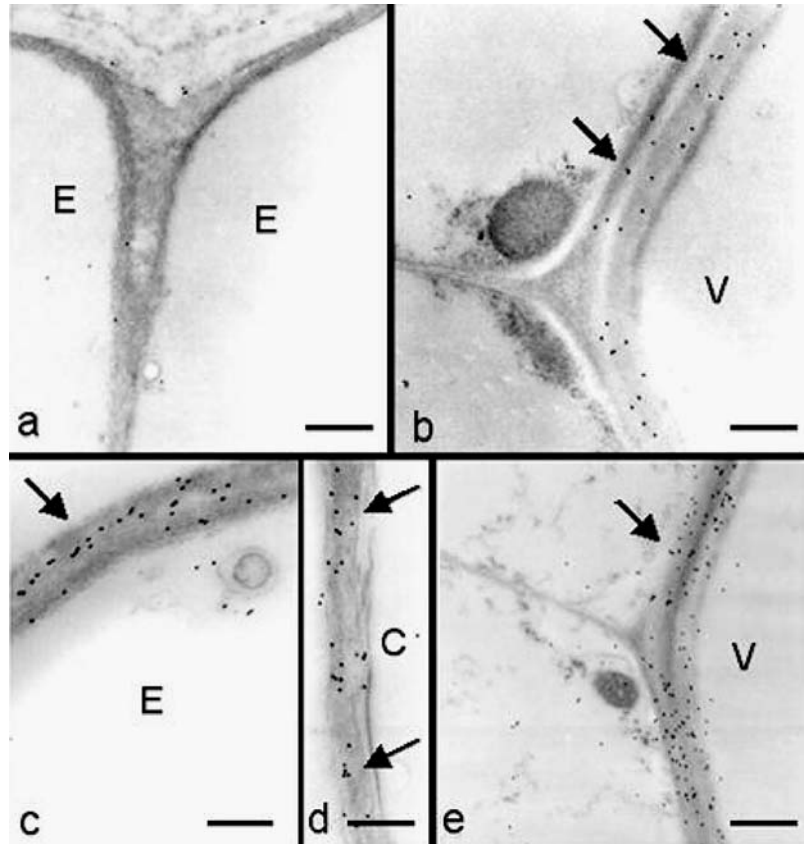


Fig. 4 Detection of expansins in non-mycorrhizal cucumber roots after immunogold labelling. Ultra-thin sections were treated with anti-EXPA1 (a, b) or anti-EXPA2 (c–e). **a** Under electron microscope, very few gold granules are evident on epidermal cell wall (E) after treatment with anti-EXPA1. Bar 0.4 μm . **b** The same treatment led to a consistent labelling (arrows) on the cell walls in vascular tissue (V). Bar 0.3 μm . **c–e** After the treatment with anti-EXPA2, an intense labelling (arrows) was present on the cell walls of all tissues: epidermis (e), cortex (d) and central cylinder (e). C cortical cell, V vessel, E epidermal cell. Bars 0.3 μm for (c, d) and 0.4 μm for (e)



the AM fungus was associated with increased cell-wall thickness (Fig. 6a, b; Table 1).

Co-localization of expansins and cellulose

Expansin may weaken the non-covalent adhesion of glucans to one another (e.g. xyloglucan to cellulose; Cosgrove et al. 2002). To localize cellulose, we used an enzyme–colloidal gold complex, a one-step technique where the enzyme binds to its substrate. The complex CBH I-gold adhered to cellulose over the plant wall. In the presence of the mycorrhizal fungus, abundant labelling was regularly found on the plant wall and in the interface zone at the penetration point (Fig. 7a, b). A limited number of gold granules or no gold granules were found around the intracellular hyphae (Fig. 7a). Since expansins bind to cellulose in vitro (McQueen-Mason and Cosgrove 1994), we carried out double-labelling experiments with anti-EXPA2, as both cellulose and anti-EXPA2 antigens were consistently present in cortical cell walls (Figs. 2, 7). Antigens recognized by anti-EXPA2 were found to be co-localized with β -1,4-glucans in the plant cell wall of the different root tissues, both in non-mycorrhizal (not shown) and mycorrhizal samples (Fig. 7c, d). A parallel double-labelling experiment by using anti-EXPA1 was not performed since this antibody mostly labels the interface where cellulose was not detected by using the CBH I-gold complex (Fig. 7).

Discussion

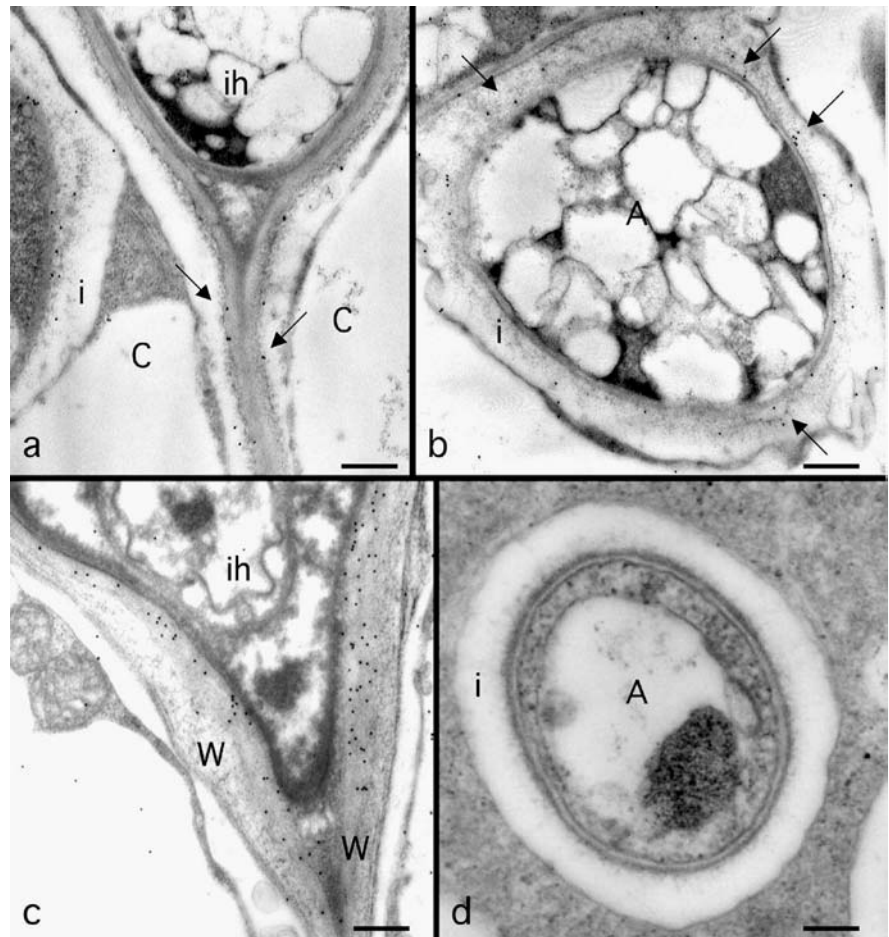
Our results demonstrate, for the first time, that the distribution of certain α -expansin isoforms, recognized by anti-EXPA1, is altered upon infection of cucumber roots by *G. versiforme* mycorrhizae. Antigens recognized by anti-EXPA1 are mostly associated with a novel compartment typical of the symbiosis, the interface, in addition to the cell walls of vascular tissue and root cap cells (R. Balestrini, P. Bonfante and D. Cosgrove, unpublished). Antigens recognized by anti-EXPA2 are associated with the cell walls of different tissues present in the differentiated region of the roots and co-localize with cellulose. The colonization process is accompanied by a previously undescribed increase in cell size and cell-wall thickness.

Taken together our results reveal new aspects of the accommodation of AM fungi by infected cortical cells (Parniske 2000; Novero et al. 2002). Our results suggest that cell-wall-loosening agents like α -expansins might be operating at the same time in order to allow the penetration of the fungal hyphae through the cell wall.

EXPA1–EXPA2: a specific localization versus a specific function in mycorrhizal cells

Several studies report the use of anti-expansin antibodies in immunoblot experiments (Li et al. 1993; Rose et al.

Fig. 5 Detection of expansins in cucumber/*G. versiforme* root sections after treatment with anti-EXPA1 (a, b) or anti-EXPA2 (c, d). **a** In the presence of the mycorrhizal fungus, a few gold granules are evident on plant cell walls by using the anti-EXPA1. C cortical cell, ih intercellular hypha, i interface. Bar 0.35 μm . **b** Gold granules (arrows) are present in the interface space (i) around the intracellular fungus. A arbuscular branch. Bar 0.35 μm . **c** After treatment with anti-EXPA2, an important labelling is detectable on cortical cell walls (W) around the intercellular hyphae (ih). Bar 0.35 μm . **d** No labelling can be seen in the interface zone (i). A arbuscular branch. Bar 0.14 μm



2000; Wu et al. 1996; Link and Cosgrove 1998). By use of an antibody raised against CsEXPA1 from cucumber hypocotyls, Harrison et al. (2001) identified two bands, corresponding to 29 and 31 kDa, respectively, in an immunoblot of proteins extracted from developing strawberry fruit. The presence of expansins with different size (30 and 27 kDa) were also found in maize roots, where an enhanced expansin activity was associated with a higher abundance of α -expansin proteins (Wu et al. 1996). Little information is available about the sub-cellular and tissue localization of expansins. Zhang and Hasenstein (2000) reported the presence of α -expansins on the root cap cell wall, suggesting a role in the sepa-

ration process during the sloughing of root cap cells. Antigens recognized by the anti-EXPA1 are also present on the cap cell wall in cucumber root tip. We also observed labelling of the cell walls of vascular tissue, consistent with the results obtained by Cho and Kende (1998) in deepwater rice internodes.

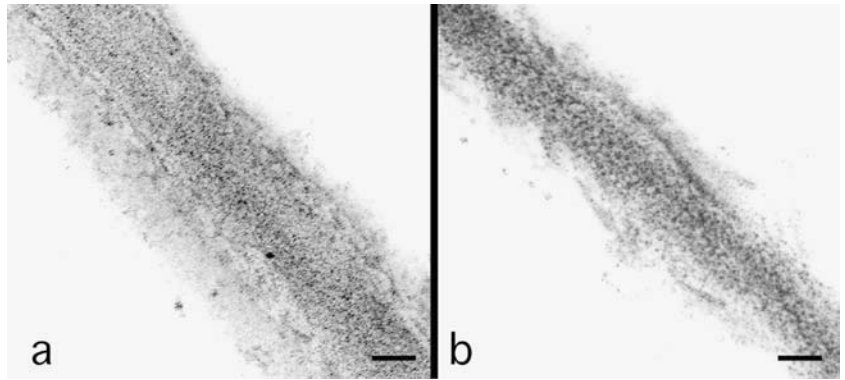
Our experiments reveal that different expansins occupy distinctive locations: epitopes recognized by anti-EXPA2 are detectable on the walls of all the root cell types, irrespective of the fungal presence. Epitopes recognized by anti-EXPA1, in contrast, were closely related to the presence of the symbiont, also being located at the interfacial compartment. Interestingly, in our immunoblot experiments, anti-EXPA1 revealed an additional band, which might correspond to an expansin induced by the presence of the AM fungus. The fact that, in immunogold experiments, gold granules were not present on the fungal wall supports the idea that the specific band present in the mycorrhizal samples is of plant origin. Its specific location in the interface suggests a role in the formation of this compartment, which is characteristic of endomycorrhizae. The interface mostly contains plant-derived cell-wall molecules; however, its morphology indicates that the wall components are not fully assembled into a dense cell wall since they show a loose appearance (Bonfante 2001). EXPA1 or related

Table 1 Mean size and cell wall thickness (\pm SD) of root cortical cells in the presence or absence of the arbuscular mycorrhizal fungus

	Length of cortical cells (μm)	Width of cortical cells (μm)	Cell wall thickness (μm)
Non-mycorrhizal root	56.6 \pm 27	21.2 \pm 10	0.13 \pm 0.02
Mycorrhizal root	82.7 \pm 35	28.0 \pm 12	0.23 \pm 0.06

Values of each columns are significantly different according to Kruskal-Wallis test ($P < 0.01$)

Fig. 6 Magnification of cortical cell wall from mycorrhizal (a) and non-mycorrhizal (b) roots. PATAg reaction for polysaccharide detection. The magnification clearly shows the difference in thickness. Bars 0.1 μm



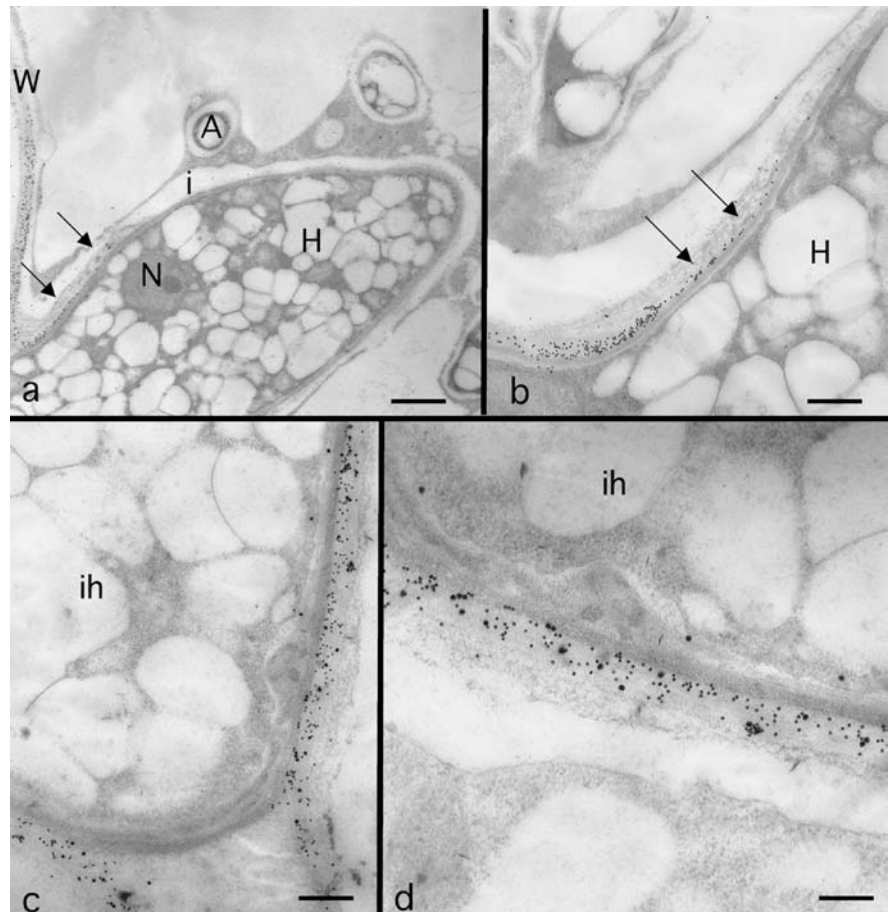
α -expansins could be involved in keeping the interfacial material loose.

Immunoblot and immunolabelling experiments demonstrate the abundant presence of epitopes recognized by anti-EXPA2 on the walls of root cells. Since morphometric analyses have revealed an increase in the size of arbuscule-containing cells, we hypothesize a role of EXPA2 (or related α -expansins) in these events of cell expansion and cell-wall extension. α -Expansins could be involved in cell enlargement and cell-wall loosening, which are both required during the intracellular colonization.

Expansins and cellulose during cell-wall expansion

The enlargement of plant cells requires slippage of the structural polymers within the cell wall, which must simultaneously maintain sufficient strength to withstand high-turgor forces. Expansins can disrupt hydrogen bonding between cellulose fibres (McQueen-Mason and Cosgrove 1994) and their action in the growing cell wall is likely to catalyse the slippage between cellulose microfibrils and the polysaccharides matrix, and thereby catalyse wall stress relaxation, followed by wall surface expansion and plant cell enlargement (McQueen-Mason

Fig. 7 Localization of cellulose (a, b) and cellulose/expansins (c, d) in ultra thin sections of a mycorrhizal root. a, b After treatment with CBH I-colloidal gold complex, labelling (arrows) is localized on the interfacial material at the penetration point. A arbuscular branch, N nucleus, i interface, H penetration hypha, W plant cell wall. Bars 1.1 μm for (a) and 0.5 μm for (b). c, d In double-labelling experiments, expansins recognized by anti-EXPA2 (10 nm gold granules) are co-localized with cellulose (20 nm gold granules) on the plant cell wall. ih intercellular hyphae. Bars 0.4 μm for (c) and 0.25 μm for (d)



and Cosgrove 1994). Evidence for this is seen in its weakening effect on pure cellulosic paper (a hydrogen-bonded network of glucans) without evidence of hydrolytic activity. This hypothesis has also been tested with a composite material consisting of crystalline cellulose produced by *Acetobacter xylinus* in the presence of hemicellulose polymer. Purified cucumber expansin induced the extension of cellulose-xyloglucan composites under constant load to the same extent as it induces extension of plant cell wall. The results with this “artificial cell wall” suggest that expansin affects the tethering of cellulose microfibrils to one another via xyloglucan bridges (Whitney et al. 2000). In our study, by use of a CBH I–gold complex in combination with anti-expansin antibodies, we show that expansins are co-localized with cellulose along the cell wall of root cells, including those containing the fungus. The observation suggests that the cell-wall extension measured in cucumber mycorrhizal cells as size increases may be the result of a direct interaction between EXPA2 (or related α -expansins) and the pre-existing cellulose.

Candidate enzymes responsible for the re-organization of the cellulose-xyloglucan framework include XETs that cleave xyloglucan chains and attach the cut ends to new xyloglucan acceptors (Fry 1995). XET is thought to be involved in cell-wall remodelling during cell growth. However, it is not known how this is coordinated with wall synthesis. A relationship between cellulose synthesis and cell expansion is reported by Pagant et al. (2002). Interestingly, they show that a new locus, KOBITO1 (KOB1), which was identified by mutations producing a defect in the synthesis of cellulose during the elongation phase of the cell, encodes a novel plasma membrane-anchored protein that may be a part of the cellulose synthesis machinery and may play a role in the co-ordination between cellulose synthesis and cell expansion. Experiments that tested for wall loosening by XET did not support its proposed role as a primary wall-loosening agent (McQueen-Mason et al. 1993), but XET action might modulate the sensitivity of the cell wall to expansin action (whether positively or negatively is not known).

The increased thickness of the cell wall after mycorrhizal infection could be the result of the loosening required for the fungal intracellular colonization (Bonfante 2001). Alternatively, it could derive from synthesis and deposition of new cell-wall polymers.

Expression of cell-wall-related genes in mycorrhizal cells

A recent paper on cDNA arrays shows the transcript profile in *M. truncatula* roots during the development of an AM symbiosis with *G. versiforme* (Liu et al. 2003). It reports the up-regulation of different cell-wall genes but no genes coding expansins were detected. Recently, an up-regulated gene coding for an α -expansin was found in mycorrhizal roots by EST analysis (<http://medicago.toulouse.inra.fr/EST>). Preliminary

experiments in RT-PCR demonstrated that expansin transcripts are present in cucumber mycorrhizal roots, like in non-mycorrhizal ones (R. Balestrini and P. Bonfante, unpublished). The difficulty in analysing the steady-state level of expansin genes could be due to the fact that the mycorrhizal process is asynchronous and information concerning the spatial expression pattern cannot be obtained from these analyses (Liu et al. 2003).

In situ analyses seem to be more informative, allowing the demonstration that cell-wall genes are induced in mycorrhizal roots and that transcripts are localized specifically in the cells containing arbuscules (Balestrini et al. 1997). Moreover, α -expansins comprise a large multigene family whose members show diverse organ-, tissue-, and cell-specific expression patterns. Since the immunolocalization studies were made with polyclonal antibodies that recognize numerous (but not all) EXPAs, it is possible that the signal derives from multiple EXPAs, and that other EXPAs are present but not recognized by the antibody (Cosgrove et al. 2002). Because of the large sequence divergence between EXPA and EXPB proteins, the antibodies used in this study do not recognize EXPB epitopes.

Irrespective of that, expression of genes which could be involved in the further steps has been well demonstrated. It is interesting that two XET genes have been isolated from *M. truncatula*, one being expressed only in mycorrhizal roots. According to Maldonado-Mendoza and Harrison (1998), XETs may be involved either in facilitating hyphal penetration and arbuscule formation by allowing localized cell-wall loosening, or in modifying the structure of XG in the interface compartment. Xyloglucanase activity has been observed previously in mycorrhizal roots; however, XET activity has not been reported. Recent results of the same group (Liu et al. 2003) show that the product of one mycorrhizal-induced gene (*MtCell*) shares identity with members of the E-type of endo- β -1,4-glucanase subfamily III. Experiments with transgenic roots (carrying *MtCell* promoter-GFP fusion or *MtCell* promoter-GUS fusion) found reporter gene expression only in cells containing arbuscules, suggesting that MtCEL1 is located in the periarbuscular membrane and involved in the assembly of cellulose/hemicellulose matrix. The analysis of molecules involved in cellulose synthesis would provide new insights into the possibility that the cell-wall extension in the cells containing the AM fungus could be related to new polysaccharides synthesis.

In conclusion, our results, which are the first on the presence of α -expansins associated with mycorrhizal cells, suggest that the fungus induces cortical cell enlargement via the activity of α -expansins. This represents a crucial piece of the puzzle concerning plant cell-wall re-organization in mycorrhizal cells. Previous papers have demonstrated that this re-organization is also related to a quick cytoskeleton re-arrangement (Blancaflor et al. 2001; Genre and Bonfante 2002). It will be very interesting to understand whether the cell-wall

loosening, which seems to be expansin-mediated, also modulates the cytoskeleton re-organization.

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