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IMMUNOLOCALIZATION OF α-EXPANSIN PROTEIN (NTEXPA5) IN TOBACCO ROOTS IN THE PRESENCE OF THE ARBUSCULAR MYCORRHIZAL FUNGUS *GLOMUS MOSSEAE* NICOL. & GERD.

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The arbuscules of mycorrhizae develop within apoplastic compartments of the host plant, as they are separated from the cell protoplast by an interfacial matrix continuous with the plant cell wall. Expansins are proteins that allow cell wall loosening and extension. Using fluorescence and electron microscopy we located the NtEXPA5 epitopes recognized by polyclonal antibody anti-NtEXPA5 in mycorrhizal tobacco roots. The expansin protein was localized mainly within the interfacial matrix of intracellular hyphae, arbuscule trunk and main branches. NtEXPA5 proteins were detected neither within the interface of collapsing arbuscule branches nor in non-colonized cortex cells. In plant cell walls, expansin protein was detected only at the penetration point and in the parts of cell walls that adhered firmly to fungal hyphae growing intracellularly. For the first time, NtEXPA5 protein was localized ultrastructurally in hyphae growing intracellularly at the interface of the hypha tip and sites of bending. The novel localization of NtEXPA5 protein suggests that this protein may be involved in the process of arbuscule formation: that is, in promoting apical hyphal growth and arbuscule ramification, as well as in controlling the dynamic of arbuscule mycorrhiza development.

Key words: Arbuscular mycorrhizae, arbuscule, expansin, host/fungus interface, ultrastructure.

INTRODUCTION

Arbuscular mycorrhizae (AM) are the most common root-fungus symbiotic association, formed by fungal species of the Glomeromycota (Brundrett, 2004). As the AM association develops, hyphae of arbuscular mycorrhizal fungi (AMF) penetrate the cell walls and develop within the cortex cells by repeated dichotomous branching of intracellular hyphae, forming tree-like structures called arbuscules. Arbuscules are the region of two-way nutrient exchange. AMF supply the plant cell with macro- and microelements, mainly phosphorus, in return for photosynthetically fixed carbon (Peterson et al., 2004; Smith and Read, 2008). Arbuscules are short-lived structures; after a few days of metabolic activity an individual arbuscule degenerates, collapses, and forms a clump (Cox and Tinker, 1976; Alexander et al., 1988, 1989). More than a century has passed since the first detailed descriptions of AM structures were published (Gallaud, 1905) but we are only beginning to understand the mechanisms that control their development, including the potential role of the host plant apoplast. It is clear that colonization of cortex cells, ramification of intracellular hypha and arbuscule formation require fungal signal identification and molecular regulation (Reinhardt, 2007). Eventually this signaling pathway alters gene expression and leads to specific changes in cell organization and metabolism. Most of the activated genes are expressed exclusively in the root cells that contain arbuscules, among them genes that have a function in cell wall modification (Balestrini and Bonfante, 2005).

Arbuscules occupy a large part of the plant cell volume but they are separated from the cell protoplast by the periarbuscular membrane (PAM) originating from the host plasma membrane (Gianinazzi-Pearson et al., 1991; Raush et al., 2001; Harrison et al., 2002). The resulting space between the PAM and the fungal plasma membrane consists of an apoplastic compartment which represents the symbiotic interface. The interfacial matrix, situated between the fungal cell wall and the PAM, continuous with the plant cell wall, is composed of cellulose, pectins, xyloglucans, nonesterified polygalacturonans, ara-

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binogalactans and hydroxyproline-rich glycoproteins (HRGP) (Bonfante and Perotto, 1995; Golotte et al., 1996, 1997; Armstrong and Peterson, 2002). Presumably the development of this apoplastic compartment is the most important event marking successful colonization, as it is thought to be the main site of nutrient exchange between the fungal and plant symbiotic partners (Bonfante and Perotto, 1995). To our understanding, the components of the interface matrix must play a key role not only in nutrient transfer but also in controlling the spread of the infecting fungi inside the root cells. Our work is intended to shed light on these roles by examining the potential influence of expansins, extracellular proteins modifying the cell wall matrix.

Expansins are proteins that play a crucial role in cell wall loosening and extension, encoded by a superfamily of genes whose products are grouped in four families: α-expansins (EXPAs), β-expansins (EXPBs), and expansin-like A (EXLA) and expansin-like B (EXLB) proteins (Cosgrove, 2000; Kende et al., 2004, Choi et al., 2006). Expansins are ~27 kDa and ~225 aa proteins consisting of two compact domains: the N-terminal, which resembles the catalytic domain of family 45 glycoside hydrolases (GH45); and the C-terminal, which has been reported as a cellulose-binding domain (Yennawar et al., 2006). Expansin genes appear to be highly conserved throughout plant evolution, but proteins remarkably similar to plant expansin sequences have also been found in *Dictyostelium discoideum*, (Li et al., 2002), nematodes (Qin et al., 2004; Kudla et al., 2005; Fudali et al., 2008), the ascomycete fungi *Trichoderma reesii* (Saloheimo et al., 1994), mussels (Xu et al., 2001) and the bacterium *Bacillus subtilis* (Kerff et al., 2008). The expansin-like sequences in bacteria, animals and fungi appear to be restricted to organisms involved in plant pathogenesis or plant cell wall digestion (Li et al., 2002). Differences in gene structure suggest that the plant expansin genes evolved separately from genes in non-plant organisms (Choi et al., 2006). Although expansins are widespread in many plants, the precise mechanism of wall loosening by expansins is still an open question. Most of the biochemical work on expansins has focused on α-expansins (EXPAs), which do not hydrolyze the major structural polysaccharides of the wall and are devoid of enzyme activity. The current model posits that EXPAs affects the stability of the hydrogen bonds that link cellulose and hemicellulose microfibrils together (McQuinn-Mason and Cosgrove, 1994; Sampedro and Cosgrove, 2005). Recent studies have provided evidence for a broad range of biological roles of expansins, suggesting that different expansins may play different roles in plant growth and development. Expression of expansin genes is correlated with internodal growth in deepwater rice (Lee and Kende, 2001, 2002), root hair formation in *Arabidopsis* (Cho and Cosgrove, 2002), root elongation in soybean (Lee et al., 2003), and fruit tissue softening (Rose et al., 1997; Hiwasa et al., 2003; Trivedi and Nath, 2004; Kitagawa et al., 2005). Whether expansins have any symbiosis-specific functions is unclear; however, an expansin gene is up-regulated in nitrogen-fixing nodules (Flemetakis et al., 2004; Giordano and Hirsh, 2004). It is known that there are a large number of structural and functional similarities between AM and N-fixing nodule formation. The assumption that AM-specific expansins exist was recently supported by studies showing that expansin and expansin-like genes are up-regulated during the early events of AMF infection (Weidmann et al., 2004, Balestrini et al., 2005, Siciliano et al., 2007, Genre et al., 2009, Dermatsev et al., 2010). These findings suggest that fungal contact and/or fungal enzymatic activity may stimulate plant cell wall plasticity. Comparison of expression profiles revealed that an expansin-like gene expressed in *Gigaspora margarita*-inoculated wild-type epidermis during PPA development can be regarded as an early host marker for successful mycorrhization (Siciliano et al., 2007). Expansin-like transcript (EXLB1) localization suggests local induction of EXLB1 transcription in AMF-tomato interaction (Dermatsev et al., 2010). Reduction of the steady-state level of the EXLB1 transcript was found to be correlated with a lower rate of infection, reduced arbuscule expansion and reduced AMF spore formation. On the other hand, immunolocalization of expansin proteins in cucumber roots in the presence of *Glomus versiforme* revealed different localization in the interface zone or in the plant cell wall, depending on which antibody was used (Balestrini et al., 2005). This indicates different functions for each analyzed expansin protein.

In light of such new data, key questions can be posed and investigated on the role of host plant expansins in arbuscule formation. Here we report our work on immunolocalization of *Nicotiana tabacum* expansin 5 (NtEXPA5) at different stages of the arbuscule life cycle.

MATERIALS AND METHODS

BIOLOGICAL MATERIALS AND GROWING CONDITIONS

Tobacco seeds (*Nicotiana tabacum* L. cv Samsun) (Plant Breeding and Acclimatization Institute, Młochów Research Center) were surface-sterilized by soaking in 70% (v/v) ethanol for 1 min and in 0.5% (v/v) sodium hypochlorite for 1 h. Seeds were germinated in Petri dishes containing 1% (w/v) agar at 20°C. Ten-day-old seedlings were transferred to pots filled with a mixture of sterile quartz sand,

expanded perlite and soil (1:1:1) containing inoculum of AM fungi *Glomus mosseae* Nicol. & Gerd. (strain deposited in the Bank of European Glomales as BEG 12). Plants were maintained in a growth room under a 14/10 h day/night cycle (22/17°C). Plants were harvested 8, 10 and 12 weeks after inoculation. Root segments from 10 independent experiments and 50 pots were analyzed.

TRANSMISSION ELECTRON MICROSCOPY

Root fragments for ultrastructure observations were fixed for 2 h with 4% (w/v) paraformaldehyde and 5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (Karnovsky, 1965), pH 7.2, under lowered pressure (-0.6 atm) and at room temperature. Samples were post-fixed with 2% (w/v) OsO_4 (same buffer) for 2 h at 40°C. The material was dehydrated by transfer through an ethanol series (10–98%). The specimens were embedded in Epoxy resin and polymerized at 60°C for 24 h. Ultrathin sections were stained with 1.2 % (w/v) ethanolic uranyl acetate and 2.6% (w/v) aqueous lead citrate (Venable and Coggeshall, 1965) and observed in a Morgagni 268D (Philips, FEI) transmission electron microscope. Images collected with a Morada digital camera were processed with GNU Image Manipulation ver. 2.6.

ANTIBODY

For Western blotting and immunolocalization experiments, polyclonal rabbit anti-NtEXPA5 antibody (New England Peptide, U.S.A.) was raised against 10-amino acid oligopeptide from the C-terminal part of the deduced amino acid sequence of NtEXPA5 protein (TSDGRTLTSN, AA:222-232, accession number AF049354).

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Cell wall protein extraction from mycorrhized (8 and 12 weeks after inoculation) and non-mycorrhized tobacco roots was performed according to McQueen-Mason and Cosgrove (1995). Extracted proteins were separated by SDS-PAGE using 4% stacking and 12% running polyacrylamide gels and blotted to a polyvinylidene difluoride membrane. Membranes were rinsed in 0.01 M Tris-buffered saline (pH 7.6) and blocked in 5% (w/v) dried nonfat milk in the same buffer overnight at room temperature. Membranes were rinsed in TBS buffer supplemented with 0.2% (v/v) Triton X-100 and 0.05% (v/v) Tween 20, incubated with polyclonal anti-NtEXPA5 antibody diluted 1:1000 with 3% bovine serum albumin (BSA) in TBS buffer, and then washed twice as described above. The blots were incubated with goat anti-rabbit antibody conjugated to alkaline phosphatase 1:20,000 in 5% (w/v) dried nonfat milk in Tris-HCl buffer for 1.5 h. Finally, the blots were washed and stained in Tris buffer (pH 9.5) containing BCIP (5-bromo-4-chloro-3-indolyl-phosphate) and NBT (nitroblue tetrazolium) (Sambrook et al., 1989).

IMMUNOFLUORESCENCE

Immunofluorescence experiments were performed on mycorrhized tobacco roots harvested 8, 10 and 12 weeks after inoculation. Root fragments were fixed in 4% (w/v) paraformaldehyde in 0.1 M MSB buffer (pH 6.9) with 0.1% (v/v) Triton X-100 for 2 h at room temperature and lowered pressure (-0.6 atm). Specimens were dehydrated through an ethanol series with 10 mM DTT and infiltrated in a mixture of BMM (butyl-methyl-methacrylate) resin and ethanol (1:3, 1:1, 3:1) and pure BMM resin. Polymerization was performed by UV irradiation for 20 h at -20°C. We used acetone to remove the BMM resin from semithin sections (3–4 μm) placed on the silane slides. Nonspecific binding sites were blocked with 3% (w/v) BSA in PBS for 1 h at room temperature. Next, sections were incubated with affinity-purified anti-NtEXPA5 antibody in PBS buffer (1:20) for 2 h in a humid chamber. Slides washed with PBS buffer with 0.05% (v/v) Tween-20 were saturated at room temperature in the dark for 3 h with goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) in PBS buffer for 1 h. At the end, sections were stained with toluidine blue, washed with PBS buffer and stained for 5 min in DAPI solution (1 μg/ml). Sections mounted in Immuno-fluore mounting medium were observed under an Olympus AX 70 Provis microscope equipped with a U-M61002a filter set and photographed using an Olympus SC35. Labelling specificity was determined by replacing the primary antibody with the pre-immune serum.

IMMUNOGOLD LABELLING

Root fragments of mycorrhized tobacco (8, 10 and 12 weeks after inoculation) were fixed for 2 h with 4% (w/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 0.1 M PBS buffer at lowered pressure (-0.6 atm) and rinsed with PBS buffer afterwards. Then the specimens were dehydrated in an ethanol series at 4°C, infiltrated in LR White and ethanol mixtures (1:3, 1:1, 3:1) and pure LR White resin, and then embedded in LR White resin at 4°C for 24 h. Formvar-coated nickel grids with ultra-thin LR White sections were placed in drops of PBS buffer. Blocking in 3% (w/v) BSA in PBS for 1 h or blocking in 3% BSA and 2% (v/v) NGS in PBS buffer were carried out. Grids were rinsed in PBS buffer with Tween-20 and Triton X-100 supplement and incu-

bated in affinity-purified anti-NtEXPA5 antibody diluted 1:20 in PBS buffer with 2% (v/v) BSA for 2 h. Pre-immune serum was used as control. Grids were washed and incubated in goat anti-rabbit antibody conjugated to 15 nm colloidal gold particles (Sigma) in PBS buffer, diluted 1:50, for 1 h. Sections washed in PBS buffer and distilled water were counterstained with 1% (w/v) uranyl acetate. Finally, the grids were rinsed in distilled water and left to dry. Immunogold-labelled sections were examined under a Morgagni 268D (FEI) transmission electron microscope. Images were collected and processed as described above. Images collected with a Morada digital camera were processed with GNU Image Manipulation ver. 2.6.

RESULTS

ULTRASTRUCTURAL OBSERVATIONS

We confined our ultrastructural study to the cortex of differentiated root regions because that is the site of arbuscule development. As the hyphae of *G. mosseae* spread mainly in the intercellular spaces, we observed its branches penetrating cortical cells. Some of these hyphae grew intracellularly, but in most cases branched repeatedly, forming arbuscules. Mature, metabolically active arbuscules, assessed on the basis of ultrastructural observations, occupied a major portion of the plant cortex cell (Fig. 1a). These arbuscules consisted of the arbuscule trunk ~4 μm in diameter, and highly branched hyphae further divisible by diameter into three classes: the primary, major arbuscule branches (~3.4 μm), the secondary ones, and the tertiary, fine branches less than 1 μm in diameter. The intracellular hyphae as well as the trunk and all arbuscule branches were separated from the host cell cytoplasm by a periarbuscular membrane continuous with the plasma membrane and by interface matrix continuous with the host cell wall. Together with the fungal cell wall and plasma membrane, these four structures formed the interface (Fig. 1b). In this way, all intracellular fungal structures were kept within the apoplastic compartment. As colonization progressed, arbuscules were observed to collapse (Fig. 1c).

TEM showed that the ultrastructure of cortical cells altered upon colonization by *G. mosseae*. The nucleus, larger than in uninfected cells, was often lobed and occupied a central position adjacent to the arbuscule trunk. Host cell cytoplasm near the fine arbuscule branches contained numerous plastids, mitochondria, osmiophilic globules and abundant endoplasmic reticulum (ER) cisternae (Fig. 1a–c). Unlike the non-arbusculated cortex cells in which a large vacuole occupied almost all of the cell

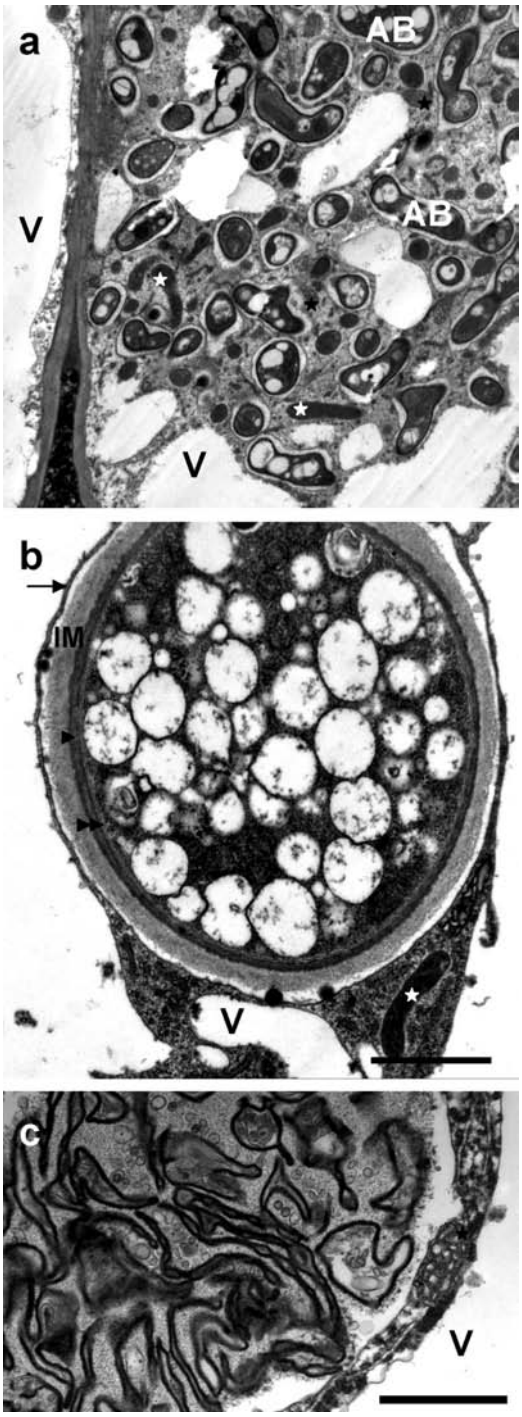

Fig. 1. Transmission electron microscopy of arbusculated cortex cells. (**a**) Mature arbuscule, showing numerous fine branches (AB) surrounded by host cytoplasm, (**b**) Detail of interface zone: periarbuscular membrane (arrow), interfacial matrix (IM), fungal cell wall (arrowhead) and fungal cell membrane (double arrowhead), (**c**) Collapsed arbuscular branches. Mitochondria (black stars) and plastids (white stars) can be seen in dense host cytoplasm surrounding arbuscule branches. Also note fragmented vacuole (V). Bar = 2 μm (a), 1 μm (b,c).

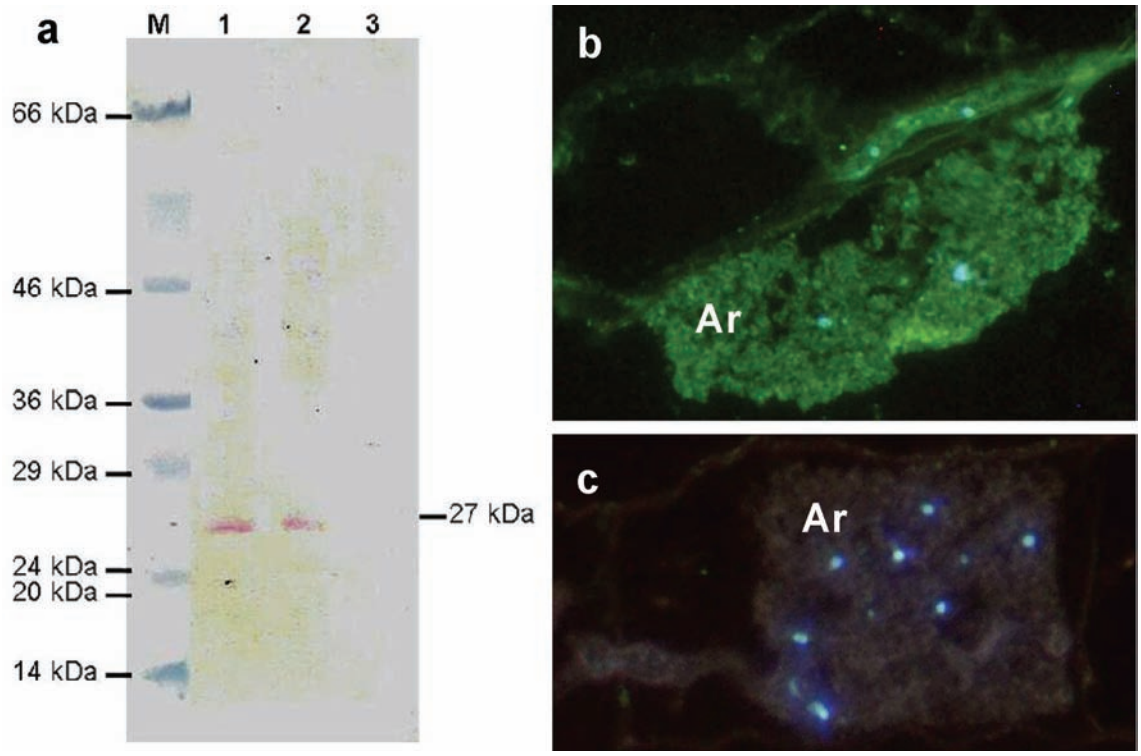

Fig. 2. (**a**) Western blot analysis of cell wall proteins extracted from mycorrhized tobacco roots 8 weeks after inoculation (1), 12 weeks after inoculation (2) and from non-mycorrhizal roots (3) with anti-NtEXPA5 antibody. The migration positions of molecular-mass markers are indicated (M). Immunofluorescence labeling of semithin sections: (**b**) Green fluorescence signal indicating the presence of NtEXPA5 protein associated with arbuscule (Ar) branches, (**c**) No signal appears in control section incubated with pre-immune rabbit serum. Plant and fungal nuclei appear blue due to counterstaining with DAPI. Bar = 50 μm.

lumen, in colonized cells the vacuolar system had undergone fragmentation (Fig. 1a). Abundant vesicles were observed in the host cytoplasm adjacent to the growing tip of intracellular hyphae.

IMMUNOLOCALIZATION OF NtEXPA5

To locate the NtEXPA5 protein at different stages of arbuscule development in the mycorrhizal tobacco roots, we used the NtEXPA5 antibody directed against part of the C-terminal amino acid sequence in immunofluorescence and immunogold experiments. The reactivity of the antibody was tested. Western blots using protein extract from mycorrhized *Nicotiana tabacum* roots (8 and 12 weeks after inoculation) with an anti-NtEXPA5 antibody identified a ~27 kDa protein (Fig. 2a), similar to the expected molecular weight of NtEXPA5. No immunoreactions were observed in the cell wall protein fraction isolated from uninfected root segments (Fig. 2a).

Immunofluorescence labeling of semithin sections was performed with a secondary antibody conjugated with FITC, giving a green fluorescence signal indicating the presence of NtEXPA5. In tobacco roots colonized by *G. mosseae* the strong fluorescence signal revealed the sites of NtEXPA5 proteins associated with well developed, highly branched arbuscules (Fig. 2b) and at the tips of hyphae growing intracellularly (Fig. 3a). NtEXPA5 was not detected in cells containing collapsed arbuscule branches (Fig. 3a), nor in non-colonized cortex cells. Control root sections incubated with pre-immune serum instead of primary antibody did not show any fluorescence (Fig. 2c).

The immunolocalization experiments on ultrathin sections confirmed the immunofluorescence results and precisely showed the sites of NtEXPA5, detected by the formation of black precipitates of colloidal gold. Labeling was associated mostly with the interface zone of intracellularly growing hyphae, in particular with their tips. Immunogold labeling showed the presence of numerous gold particles in the outer layers of the interfacial matrix around the hyphal tip (Fig. 3b,c). Many vesicles closely connected to the proliferating perifungal membrane were also labeled (Fig. 3c). Interestingly, weak gold labelling on the fungal cell wall was also noted (Fig. 3b). No gold labelling was observed after the control

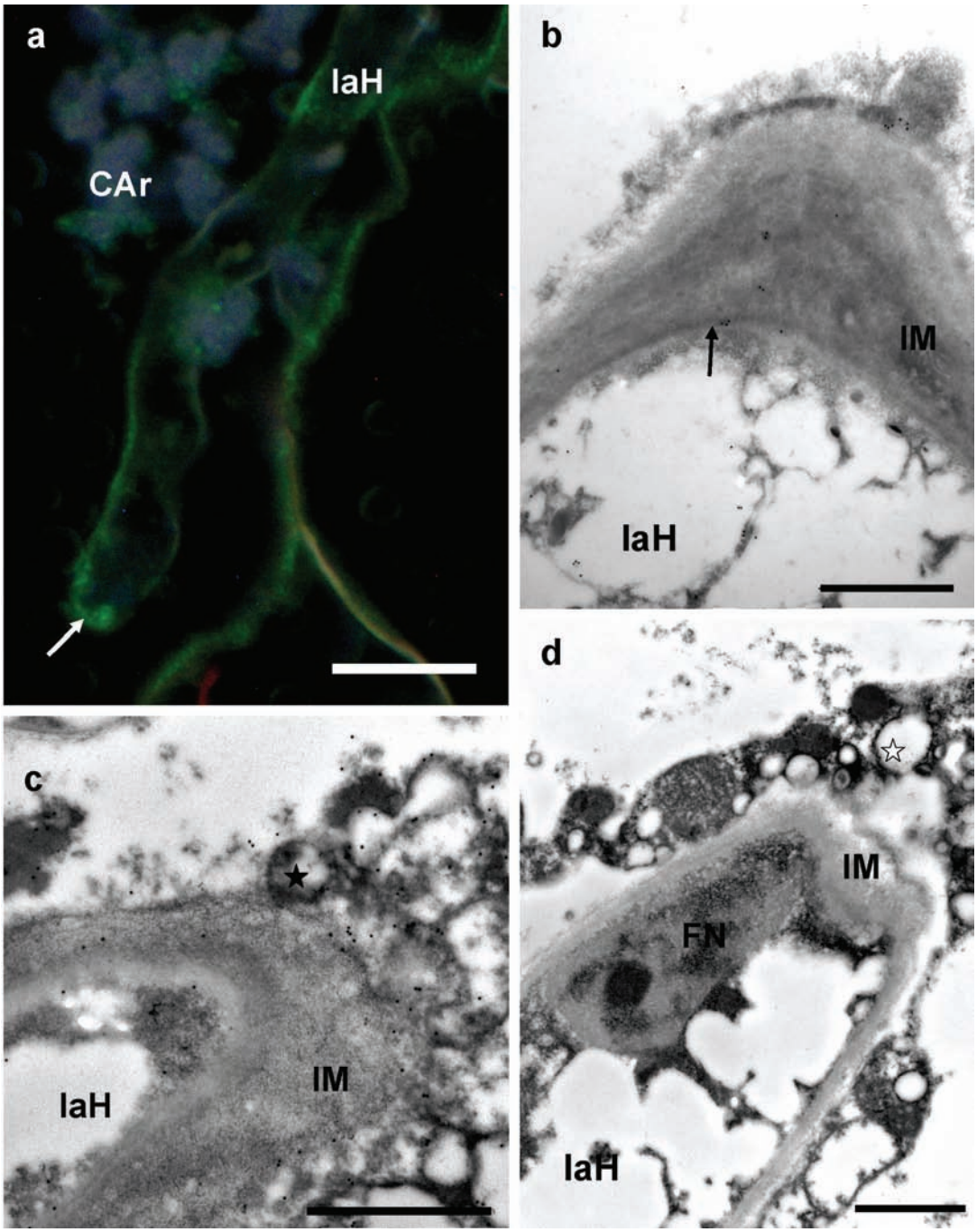

Fig. 3. Immunodetection of NtEXPA5 protein around growing tips of intracellular hyphae. (**a**) Immunofluorescence detection in semithin section shows strong green fluorescence signal (arrow) on tip of intracellularly growing hypha (IaH). Note the lack of signals around branches of collapsed arbuscule (CAr), (**b,c**) Immunogold localization of NtEXPA5 protein around growing tips of intracellular hyphae (IaH). Gold granules present on surface and inside interfacial matrix (IM); also note the presence of gold granules within fungal cell wall (arrow) and vesicles (star), (**d**) Control for immunogold localization, with antibody replaced by pre-immune rabbit serum. Fungal nucleus (FN), interfacial matrix (IM), intracellular hyphae (IaH), vesicles (star). Bar = 20 μm (a), 1 μm (b,c,d).

reaction with pre-immune rabbit serum (Fig. 3d). Gold granules indicating the presence of NtEXPA5 protein in the interfacial matrix of intracellularly growing hyphae were preferentially localized at the sites where hyphae bend (Fig. 4a) and/or changed their diameter (Fig. 4b).

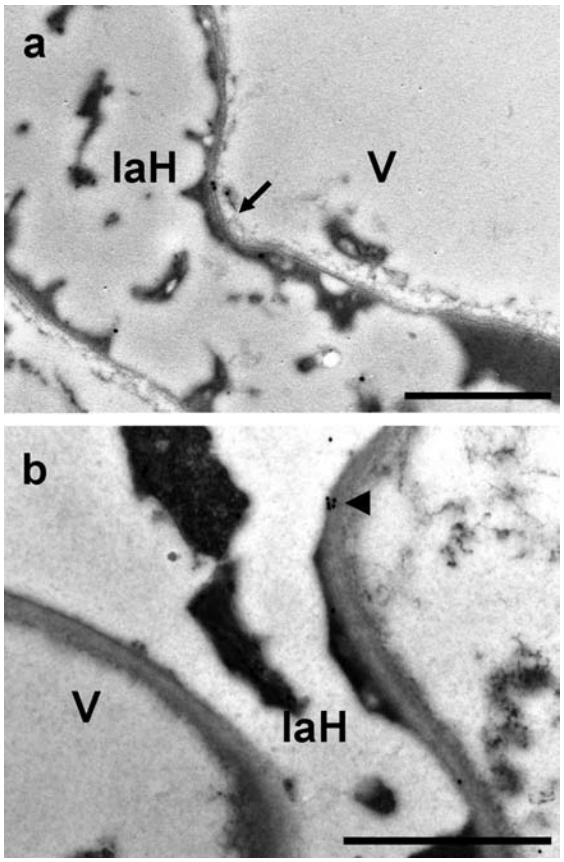

Fig. 4. Immunogold detection of NtEXPA5 protein associated with interfacial matrix around intracellular hyphae (IaH) in places of (**a**) hypha bending (arrow) and (**b**) hypha diameter change (arrowhead). V – vacuole. Bar = 1 μm.

The distribution pattern of NtEXPA5 protein associated with arbuscule branches depended on the size of the fungal branches and the stage of development. In mature, well developed arbuscules, NtEXPA5 protein was localized within the interfacial matrix of the trunk (Fig. 5a) or in primary hyphae of relatively large diameter. There was a significant difference in labeling density between primary and tertiary (fine) arbuscule branches; in the latter only single gold particles were detected (Fig. 5b). In contrast to mature arbuscule branches, immunolocalization of NtEXPA5 did not yield any signals around collapsed fungal cell walls at the stage of arbuscule degeneration; however, gold labeling was observed in host cytoplasm associated with endoplasmic reticulum (Fig. 5c).

After treatment with anti-NtEXPA5 antibody there was no labeling in plant cell walls except for two peculiar cases. Labeling of the plant cell walls was particularly abundant at the penetration point, where there was an accumulation of electron-dense material (Fig. 6a). A few gold particles indicating NtEXPA5 protein were also seen in the parts of cell walls that adhered firmly to intracellularly growing fungal hyphae (Fig. 6b).

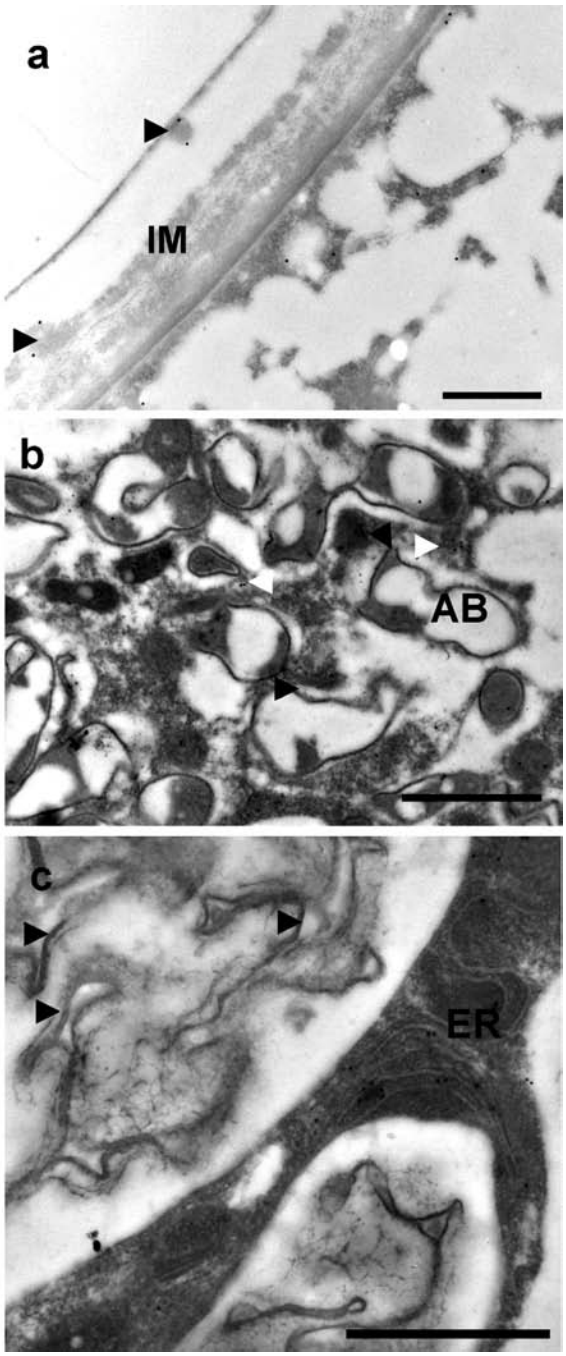

Fig. 5. Immunodetection of NtEXPA5 protein in arbusculated tobacco cortex cells. (**a**) Gold granules (arrowheads) in interfacial matrix (IM) of trunk hypha, (**b**) A few gold granules in host cytoplasm (arrowheads) around fine arbuscule branches (AB), (**c**) Immunogold labeling of ultrathin section shows absence of gold granules in vicinity of collapsed arbuscule branches (arrowhead). Note the presence of many gold granules associated with endoplasmic reticulum (ER) in plant host cytoplasm. Bar = 1 μm.

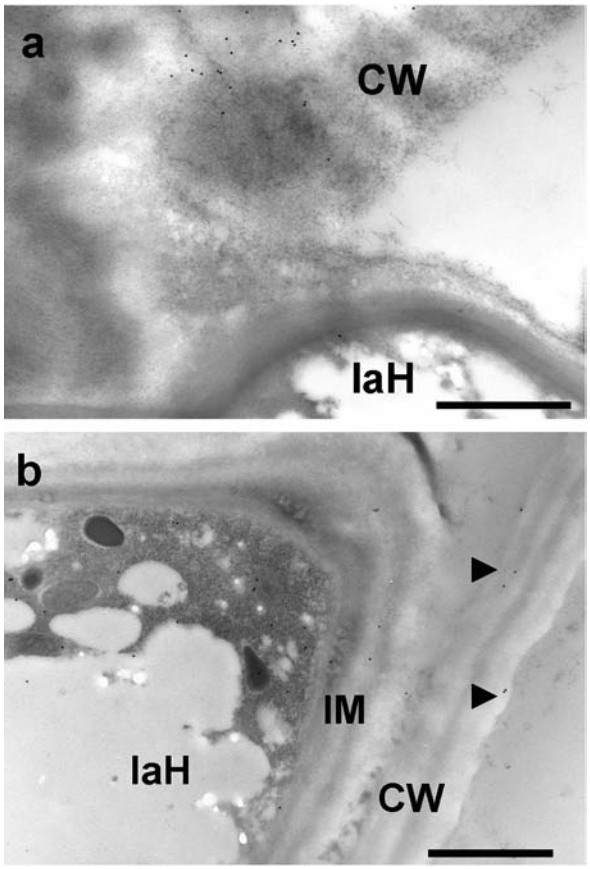

Fig. 6. Immunolocalization of NtEXPA5 protein in plant cell wall of mycorrhized tobacco cortex cells. (**a**) Gold granules on plant cell wall where intracellular hypha (IaH) penetrates, (**b**) Weak labeling (arrowheads) on plant cell wall adhering to interfacial matrix (IM) of intracellular hypha (IaH). Bars = 1 μm.

DISCUSSION

The overall appearance of the investigated mycorrhizae, including their internal structure, and especially the pattern of arbuscule development, was consistent with the general characteristics of arbuscular mycorrhizae (Smith and Read, 2008). Ultrastructural observations indicated Arum-type AM, characterized by intraradical hyphae spreading inside the root, mainly in the intercellular spaces, and usually one arbuscule developed through repeated branching of a trunk hypha. Surprisingly, relatively often we observed hyphae growing intracellularly from cell to cell.

As all intracellular fungal structures are covered by apoplastic material of plant origin, it has long been appreciated that fungal penetration of plant cell walls and arbuscule development require localized expression of proteins modifying the cell wall structure. Our results confirm that various cell wall protein-encoding genes appear to be differentially regulated upon mycorrhization. Among genes predicted to be up-regulated in mycorrhized roots are those coding for extensins, endo-beta-1,4-glucanases (Journet et al., 2002), cellulases (Liu et al., 2003), xyloglucan endo-transglycosylates (Maldondo-Mandoza and Harrison, 1998) and expansins (Weidmann et al., 2004; Siciliano et al., 2007). Endoglucanases and xyloglucan transglycosylases modify cell wall components enzymatically, whereas expansins have been identified as proteins modulating cell wall extension due to their ability to weaken noncovalent bonding between cell wall polysaccharides (Cosgrove, 1999).

The last decade has brought studies of the role of expansins in modifying the plant cell wall, both in pathogenic and in symbiotic relationships, but our knowledge of the role of expansins during mycorrhizal development is limited. Only one work had demonstrated the distribution of expansin proteins in mycorrhizal roots. Immunolocalization of certain α-expansin isoforms in cucumber roots mycorrhized by *Glomus versiforme* showed that upon AMF infection some of the expansin proteins are more abundant in cucumber cell walls, while others are localized in the interface zone (Balestrini et al., 2005). Four weeks after inoculation, expansin-like transcripts were localized to root cortical cells that were in close proximity to the colonizing fungal hyphae (Dermatsev et al., 2010). Our data are largely in agreement with these results: immunolocalization of NtEXPA5 during AM development showed NtEXPA5 proteins present exclusively in arbuscule-containing plant root cells. In other work, analysis of the expression profile of *Medicago truncatula* inoculated with *Gigaspora margarita* revealed that the gene encoding expansin-like protein is up-regulated during PPA development and is preferentially expressed in epidermal cells in contact with an AMF appresorium (Siciliano et al., 2007). The localization of the expansin-like transcript EXLB1 immediately before contact between AMF and plant roots suggests a role for plant expansins in the early stages of AMF-host interaction, affecting the ability of the fungus to proliferate (Dermatsev et al., 2010). Those observations support the suggestion of mycorrhiza-specific expansins and show a close correlations between expansin expression and AM development; they also suggest that different expansin isoforms perform distinct functions. Although AM symbiosis is considered nonspecific, it is clear that there are differences at the functional level (Smith and Read, 2008).

Expression profile analysis enables identification of novel genes, such as expansins, whose expression is regulated during the development of symbiosis. Arbuscule development in plant roots during mycorrhizal infection is highly asynchro-

nous, so the spatial distribution data provided by immunolocalization experiments is of particular importance to our understanding of the role of expansins during arbuscule establishment. Our study concentrated on the subcellular localization of NtEXPA5. For the first time, here we showed a correlation between the localization of expansin isoforms and the type of fungal hyphae. Labeling was most abundant in the matrix interface covering intracellularly growing hyphae of relatively large diameter, particularly at sites of hyphal bending and at the tips of those hyphae. This observation accords with Gooday's (1971) description of apical hypha growth. Inside the plant root cell, however, fungal hyphae are enveloped by newly created apoplastic material which functions as the site of bidirectional nutrient exchange and separates AMF hyphae from the host cytoplasm. Possibly the plant cell can influence the extent of colonization by means of the interfacial matrix. Local loosening of interface matrix due to expansin activity might facilitate hyphal spread. We suggest that the local NtEXPA5 accumulation we observed in the interface matrix surrounding the hyphae tip can be interpreted as a positive response of the plant to AM colonization within the cortical cells. The observed assemblage of vesicles close to the hyphae apices supports this interpretation, as expansins are synthesized in the Golgi apparatus and delivered to the wall through secretory vesicles. Our observations suggest that besides promoting apical hyphal growth, NtEXPA5 proteins facilitate arbuscule ramification and thereby determine the size and shape of the arbuscule and control the dynamics of arbuscule development. This idea opens up interesting possibilities for future research on AM through approaches using mycorrhiza-defective plant mutants. At this time it is completely unclear what triggers the profuse hyphal branching observed during arbuscule development.

The model of expansin action is still uncertain. In contrast to enzymatic wall loosening, it has been proposed that expansins locally weaken noncovalent binding between cellulose microfibrils and xyloglucans, causing cellulose chain movement and stress relaxation (Cosgrove, 2000; Whitney et al., 2000). In maize, β-1,4-glucans were detected around the large intracellular coils of *Glomus versiforme* but not around the thinner arbuscular branches (Balestrini et al., 1994). This is consistent with our results demonstrating the presence of NtEXPA5 inferred from immunogold labeling in the interfacial matrix surrounding the trunk and primary arbuscule branches. NtEXPA5 was detected only sporadically around tertiary arbuscule branches. Although expansin lacks hydrolytic activity by itself, it does enhance the breakdown of cellulose by fungal (Cosgrove et al., 1998) or bacterial cellulases (Kim et al., 2009). It has been suggested that this synergistic interaction is a result of expansins making the glucans on the surface of microfibrils more accessible to cellulases (Cosgrove, 2000). On the other hand, fungal pectinase enhanced α-expansin-induced wall extension in a concentration-dependent manner, suggesting that the pectin network may normally regulate the accessibility of expansin to the xyloglucan-cellulose complex (Wei et al., 2010). In this context we hypothesize that expansins are only one element of the much more complex adaptation of plant and fungal cells to arbuscular mycorrhiza.

Polyclonal antibody anti-NtEXPA5 immunolocalized a few gold particles in the fungal cell wall and single particles in the fungal cytoplasm, so the possible involvement of fungal expansin-like proteins in the process of root colonization should be investigated further. For example, our knowledge of expansin-like proteins of fungal origin is limited to one investigation (Li et al., 2002), and so far there is no evidence for the existence of AMF expansin. A single fungal expansin-like sequence was found in the ascomycete *Trichoderma reesii* (Saloheimo et al., 1994). We surmise that the NtEXPA5 protein localized inside the fungal hyphae was incorporated from the interfacial matrix.

Expansin-coding genes have been demonstrated to be up-regulated not only in AM but also during nodulation (Giordano and Hirsh, 2004). Expansin proteins have been found in the nodule apoplast as well as in infection thread walls (Sujkowska et al., 2007). Nitrogen-fixing symbiosis (NFS) and AM share a common signal transduction pathway, as microorganisms transfer mineral nutrients to the host plant in both associations, and in both associations the microorganisms are isolated from the host cytoplasm by the apoplastic compartment. As with nitrogen-fixers, expansins are necessary for successful AM development.

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