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SHORT NOTE

Localization of proton-ATPase genes expressed in arbuscular mycorrhizal tomato plants

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Abstract In arbuscular mycorrhizal symbioses, solutes such as phosphate are transferred to the plant in return for photoassimilates. The uptake mechanism is probably facilitated by a proton gradient generated by proton H^+-ATPases. We investigated expression of *Lycopersicon esculentum* Mill. H^+-ATPases in mycorrhizal and non-mycorrhizal plants to determine if any are specifically regulated in response to colonization. Tissue expression and cellular localization of H^+-ATPases were determined by RNA gel blot analysis and in situ hybridization of mycorrhizal and non-mycorrhizal roots. *LHA1*, *LHA2*, and *LHA4* had high levels of expression in roots and were expressed predominantly in epidermal cells. *LHA1* and *LHA4* were also expressed in cortical cells containing arbuscules. The presence of arbuscules in root sections was correlated with lower levels of expression of these two isoforms in the epidermis. These results suggest that *LHA1* and *LHA4* expression is decreased in epidermal cells located in regions of the root that contain arbuscules. This provides evidence of differential regulation between molecular mechanisms involved in proton-coupled nutrient transfer either from the soil or fungus to the plant.

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Introduction

Plants transport many molecules across membranes via transmembrane proteins. P-type ATPases form a large family of membrane embedded proteins that hydrolyze ATP in the process of active transmembrane transport. Recent genome sequencing efforts have revealed a total of 46 P-type ATPases in Arabidopsis and 43 in rice (Baxter et al. 2003). These ATPases have been classified into five major subfamilies, including heavy metal ATPases, Ca^{2+}-ATPases, putative aminophospholipid ATPases, a branch with unknown specificity, and H^+-ATPases. In plants, H^+-ATPases are primarily responsible for energizing the plasma membrane and generating the proton motive force (PMF) for secondary membrane transport of cations, anions, sugars, and amino acids. Through sequence homology, Baxter et al. (2003) identified 11 Arabidopsis H^+-ATPases and ten rice H^+-ATPases. It is probable that other plants have similar numbers of these genes; however, this cannot be confirmed until their genomes are fully sequenced. In *Lycopersicon esculentum* Mill. (tomato), a model plant used for mycorrhizal investigations (Smith et al. 2004), three full-length and four partial cDNA sequences have been identified (Ewing et al. 1990; Ewing and Bennett 1994). Further work has shown that some of these tomato genes are differentially regulated by the application of exogenous sugars (Mito et al. 1996), under high salt conditions (Kalampanayil and Wimmers 2001) and in the mycorrhizal symbiosis (Ferrol et al. 2002), situations that are likely to involve high levels of active transport of solutes.

H^+-ATPases are of particular interest in arbuscular mycorrhizal plants, as a functional symbiosis is characterized by bi-directional nutrient transport across a plant–fungal interface. Mycorrhizal plants have two possible pathways of nutrient uptake: (1) directly via the epidermis and root hairs and/or (2) via the mycorrhizal pathway involving uptake by the external fungal hyphae in soil, transfer to intracellular fungal structures (arbuscules or hyphal coils), and transport to the plant across the symbiotic interface(s) (see Smith and Read 1997; Smith et al. 2001). Recent physiological studies have shown that the mycorrhizal pathway may be the predominant route of phosphorous (P) uptake by plants, regardless of the responsiveness of the plant to mycorrhizal formation (Smith et al. 2003, 2004). Concurrently, molecular biological studies are increasingly revealing plant P transporters that are mycorrhizal-inducible and often associated with the periarbuscular membranes (Rausch et al. 2001; Harrison et al. 2002; Paszkowski et al. 2002; Nagy et al. 2005). These transporters, together with those in the external mycelium (Harrison and van Buuren 1995; Maldonado-Mendoza et al. 2002), are likely to be involved in the mycorrhizal pathway of P uptake.

The P transporters are thought to operate in conjunction with H^+-ATPases to generate the necessary PMF for inward transport of P (Schachtman et al. 1998). Research towards elucidating the sites and mechanisms of transport in mycorrhizal roots has included studies of H^+-ATPases as markers for energized membranes at the symbiotic interfaces that may be involved in nutrient transfer. Following the work of Marx et al. (1982), Gianinazzi-Pearson et al. (1991) used cytochemical techniques to demonstrate strong activity on the plant plasma membrane that surrounds fungal arbuscules in the root cortical cells of *Allium cepa* L. (leek). Benabdellah et al. (1999) isolated membrane vesicles from mycorrhizal and non-mycorrhizal tomato roots and observed increased plasma membrane H^+-ATPase activity in the mycorrhizal roots. This suggests that the fungus might increase expression of ATPase genes or up-regulate the activity of the H^+-ATPase proteins. Increased accumulation of H^+-ATPase gene transcripts in mycorrhizal roots was supported by RNA gel blot analysis in barley (Murphy et al. 1997). More recently, Gianinazzi-Pearson et al. (2000) used promoter-*uid*A fusion assays of *Nicotiana plumbaginifolia* ATPases to show that the promoters of two of these genes (*pma2* and *pma4*) were active in arbuscule-containing cortical cells. These results suggest that mycorrhizal colonization induced the expression of two genes responsible for the de novo H^+-ATPase activity observed in the periarbuscular membrane of mycorrhizal plants.

In this study, we have investigated the expression patterns of the known family of H^+-ATPases in tomato under both mycorrhizal and non-mycorrhizal conditions. RNA gel blot analysis was used to determine expression patterns in whole root and RNA in situ hybridization localized transcripts to specific cell types within the roots.

Materials and methods

Plant growth and inoculation

The near-synchronous nurse-pot colonization method of Rosewarne et al. (1997) was used to inoculate tomato with the mycorrhizal fungus *Glomus intraradices* Schenck and Smith DAOM 181602. Surface-sterilized seeds of tomato were grown in a seedling tray for 2 weeks before transplanting into 'nurse' pots. The nurse pots consisted of 12 leek plants grown for 6 weeks in soil inoculated with the mycorrhizal fungus. Uninoculated nurse pots were also used to generate non-mycorrhizal plants. Nurse pots were 11 cm in diameter and contained 1.4 kg of the soil mix. Plants were harvested at two time points, 4 and 10 days after transplanting (DAT). A sub-sample of each root system was taken to determine mycorrhizal colonization and the rest frozen (−80°C) for RNA extraction. A sterile mixture of washed river sand and Mallala soil [collected from Mallala, South Australia and contained 12 mg kg^{-1} bicarbonate extractable phosphate (Colwell 1963)] was used in a 9:1 ratio.

Microscopic examination

Roots were cleared (10% KOH) and stained with trypan blue as previously described (Rosewarne et al. 1997). Colonization was determined by the line intersect method of McGonigle et al. (1990) under 100× magnification. Intercellular hyphae, arbuscules, and vesicles were scored, each as a percentage of total root intersects.

DNA gel blot hybridization

Full-length cDNA fragments of *LHA1*, *LHA2*, and *LHA4* (50 ng) were run in 1.6% agarose gels in TAE buffer and blotted to Hybond N membranes (Sambrook et al. 1989). [^{32}P] dCTP-labeled probes were synthesized with the 'Ready-To-Go' DNA labelling kit (Amersham Biosciences, Castle Hill, NSW, Australia) and unincorporated nucleotides removed with ProbeQuant G-50 micro columns (Amersham Biosciences). Probes were hybridized to the membranes in a formamide buffer at 42°C according to Rosewarne et al. (1999). After hybridization, membranes were washed twice for 20 min at 65°C in the following solutions: 2× saline-sodium citrate (SSC; 0.3 M sodium chloride, 0.03 M sodium citrate pH 7.0), 0.1% sodium

dodecyl sulfate (SDS); 1× SSC, 0.1% SDS; 0.1× SSC, 0.1% SDS.

RNA gel blot analysis

RNA was extracted from the roots according to Rosewarne et al. (1999). Four replicate membranes were made from separate RNA extractions from the two different time points. Total RNA samples (10 μg) were run under denaturing conditions in 1% agarose gels and transferred to Hybond N+ nylon membranes as described in Rosewarne et al. (1999). Hybridizations and subsequent washes were carried out as described above.

Each membrane was hybridized with the probes listed below and stripped between hybridizations by washing in a freshly boiled solution of 0.05× SSC and 0.01 M ethylenediaminetetraacetic acid for 30 min. Each membrane was hybridized with full-length clones of *LHA1*, *LHA2* (Ewing et al. 1990), and *LHA 4* or with partial sequences of *LHA3*, *LHA5/6* and *LHA7* (Ewing and Bennett 1994) and a ribosomal probe from the 18S ribosome of tomato. The signal from the 18S ribosomal probe (representing loading of RNA in each lane) was used to normalize signals generated by the *LHA* genes. The mean and standard error of the normalized signal intensity were calculated from the values obtained from the four blots. Data of signal intensities were collected on a Molecular Dynamics Storm 860 gel and blot imaging system and analyzed with ImageQuant software (Amersham Biosciences).

In situ hybridization

For in situ hybridization, we utilized the full-length probes of *LHA1*, *LHA2* and *LHA4*. The procedure was identical to that described in Rosewarne et al. (1999) except that changes were made with regard to detection of the probe. After the stringent washes, the slides were washed in blocking solution [100 mM Tris–HCl, 150 mM NaCl, 0.5% blocking reagent (Roche Diagnostics, Castle Hill, NSW, Australia) pH 7.5] for 30 min at room temperature. A 1:500 dilution of alkaline phosphatase-conjugated sheep antiDIG antibody in blocking solution was pipetted onto the slides (100 μl) and a coverslip applied to each slide to ensure contact of the solution across the surface of the slide. Slides were incubated for 1 h at 37°C in a moist chamber. Coverslips were removed, and the slides were washed three times for 10 min in washing buffer (100 mM Tris–HCl, 150 mM sodium chloride, 0.05% Tween 20, pH 7.5) and twice for 10 min in detection buffer (100 mM Tris–HCl, 100 mM sodium chloride, 10 mM magnesium chloride, pH 8.0). Each slide was covered with 100 μl of 25 μg/ml Fast Red TR (Roche Diagnostics) and 1/100 dilution of HNPP (2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate; Roche Diagnostics) in detection buffer, a coverslip applied, and incubated for 30 min at room temperature. The coverslips were again removed, and the slides were washed in washing buffer. The Fast Red/HNPP incubation and wash were repeated three times. Slides were washed in water for 10 min and air-dried in the dark. Slides were mounted with 40 μl of 50% glycerol, 2% DABCO (Sigma-Aldrich, Sydney, NSW, Australia) in phosphate-buffered saline. Sections were examined using a Leica TCS SP2 confocal microscope with 488-nm excitation from the Ar laser. Green auto-fluorescence from 500–535 nm, and HNPP/Fast Red TR fluorescence from 600–650 nm were collected simultaneously in separate channels, and the transmitted differential interference contrast (DIC) image was collected in a third channel. Background noise was reduced by averaging four times for each image.

Statistical and sequence alignment analysis

To determine the significance of the relationship between probe signal and presence of arbuscules, the data were modeled using the GENMOD procedure of SAS (SAS Institute 2000, SAS/STAT®, Version 8.2, Cary, NC, USA) with the logit function as the link and evaluated by χ^2 analysis. Test for significance between means of hybridization signals was determined by the Student's *t* test using a two-tailed distribution and homoscedastic variance. Sequence alignments were conducted with the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) using ClustalW multiple alignment and sequence identity matrix commands to determine nucleic acid sequence identities of overlapping sequences.

Results

Biological material

Near-synchronous colonization of tomato roots by *Glomus intraradices* occurred in the nurse pots. Levels of both total colonization and formation of arbuscules in the mycorrhizal roots increased over the 10-day period (Table 1). High numbers of developmentally similar arbuscules were present in the roots at 10 DAT, enabling study of plant genes regulated in response to arbuscule formation. Control plants did not become colonized (results not shown).

DNA gel blot hybridization

The ATPase genes used in this study show high levels of nucleic acid sequence identity. To rule out any issues associated with cross-hybridization, we used each of the full-length cDNA sequences as probes against themselves

Table 1 Percent colonization of mycorrhizal roots at 4 and 10 DAT into nurse pots with actively growing fungal mycelium

Days after transplantation (DAT)	Total colonization	Arbuscular colonization	Vesicular colonization
Day 4	2.8 (1.9)	0	0
Day 10	60.9 (4.1)	21.5 (1.7)	14.4 (5.4)

Data show means (±SE) from four replicate pots.

(Fig. 1), and there was no significant cross-hybridization under the stringency conditions applied.

Expression of P-type H^+-ATPase genes

Expression of plant P-type H^+-ATPase genes was analyzed in response to mycorrhizal colonization at 4 and 10 DAT (Fig. 2). Three of the seven known *L. esculentum* H^+-ATPase genes had high levels of expression in RNA gel blots. These were *LHA1*, *LHA2*, and *LHA4*. Expression of other previously characterized members of this multi-gene family (*LHA3*, *LHA4*, *LHA5/6*, and *LHA7*) were undetectable in RNA gel blots of mycorrhizal root RNA (data not shown). Consequently, these LHA members were not analyzed further.

RNA gel blots were replicated (four identical blots) to facilitate the calculation of means, standard errors, and statistical tests for significance (Fig. 2). Expression of *LHA1* was higher in mycorrhizal plants when compared to non-mycorrhizal plants at 4 DAT ($P<0.05$). However, the expression in mycorrhizal plants had significantly decreased by 10 DAT ($P<0.01$), whereas expression of *LHA1* in non-mycorrhizal plants over the same time frame did not change. The level of *LHA2* expression did not change between mycorrhizal and non-mycorrhizal treatments, nor over time. Expression of *LHA4* somewhat mirrored *LHA1*, with a drop in expression in the mycorrhizal roots between the first and second harvest ($P<0.01$), whereas not altering in the non-mycorrhizal samples over the same time period. The finding that expression of *LHA1* and *LHA4* was lower in roots containing large numbers of arbuscules than in non-mycorrhizal roots of the same age was confirmed with another independent biological sample (data not shown).

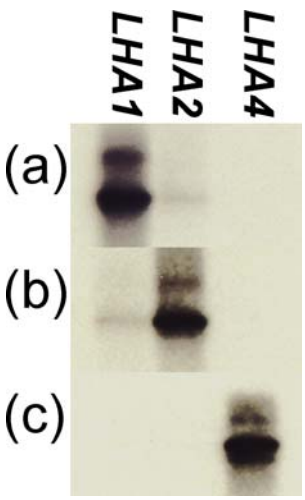

Fig. 1 DNA gel blots of full-length cDNA probes against themselves, showing a lack of cross-hybridization between the three cloned sequences. Replica blots of 50 ng of *LHA1*, *LHA2*, and *LHA4* cDNAs were probed with [^{32}P] dCTP labeled **a** *LHA1*, **b** *LHA2*, and **c** *LHA4*. Final stringency washes consisted of 0.1× SSC, 0.1% SDS at 65°C, twice for 20 min

RNA localization

Expression of LHA genes was examined in mycorrhizal root sections by in situ hybridization (Figs. 3, 4 and 5). Arrangement of cell layers and presence of mycorrhizal colonization are shown in Fig. 3a and b. The trypan blue-stained free-hand section (Fig. 3a) shows the structure of a fresh root section with the epidermal layer intact. This cell layer became somewhat collapsed during the processing of sections for in situ hybridization, as shown in the differential interference contrast image of a root section (Fig. 3b). Collapse of the epidermal layer was common throughout most of the sections, but did not affect hybridization of antisense signals in these cells; Fig. 3c shows intense staining when probed with the *LHA1* antisense probe.

All of the three antisense probes hybridized strongly to epidermal cells of the root, whereas the sense probes did not generate a significant signal (Fig. 4). However, there appeared to be a variation in the degree of hybridization among sections with the antisense probes in that although many sections had the majority of the epidermal cells generating a strong signal, other sections only had a small proportion of these cells generating a signal. The hybridization process was completed four times with each of the probes, and the different levels of hybridization among sections were consistently observed. The sense and antisense sections from the individual probes shown in Fig. 4 were taken from within 50–100 μm of each other.

In some of the sections, the antisense probes of *LHA1* and *LHA4* hybridized to cortical cells that contained arbuscules (Fig. 5). However, the fungal components of the arbuscules gave a strong green auto-fluorescence, often making hybridization signals difficult to detect and quantify. Although this data supports the expression of these genes in arbuscule-containing cortical cells, it was deemed to be of insufficient quality to quantifiably assess expression levels.

To quantify the level of signal generated in the epidermis, two parameters were used to score individual root sections, namely, whether the sections contained arbuscules, and whether there was heavy staining of the epidermis of the root sections. Sections were scored positive for arbuscules if they contained one or more arbuscules, and scored positive for epidermal staining if

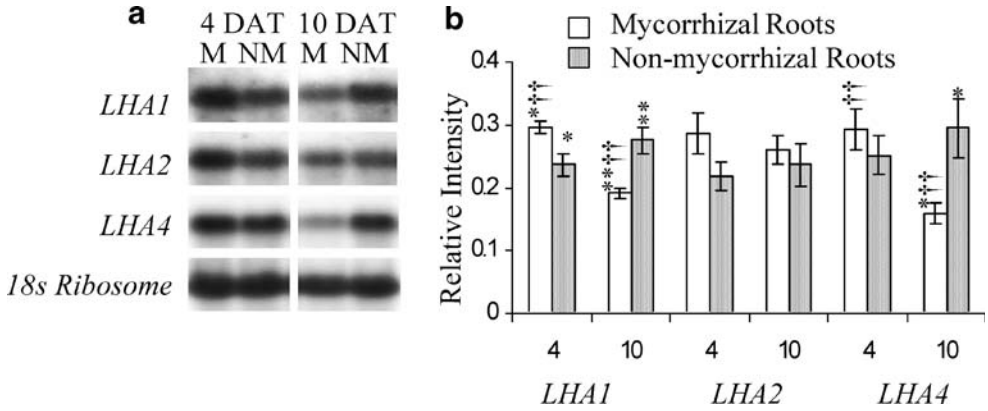

Fig. 2 Time course of expression: **a** RNA gel blots of *LHA1*, *LHA2*, *LHA4*, and the *Lycopersicon esculentum 18S* ribosomal gene in mycorrhizal (*M*) and non-mycorrhizal (*NM*) *L. esculentum* roots at 4 and 10 days after transplantation (DAT) into mycorrhizal or non-mycorrhizal nurse pots. **b** Plots of means and standard errors of RNA signal intensity from four replica blots. Signal intensity was normalized with respect to the signal generated by the ribosomal probe. *($P<0.05$) and **($P<0.01$) indicate significant differences between levels of expression of mycorrhizal and non-mycorrhizal roots at the same time point, ††($P<0.01$) significant differences in expression levels between mycorrhizal roots of different time points. Tests for significance were only conducted between signals generated from the same probe

they had at least 25% of the epidermis stained with the label. The data from four hybridization experiments were pooled. The fractions of sections containing strong epidermal fluorescence were: *LHA1* 129/285 sections positive (45.3%); *LHA2* 167/346 (48.3%); and *LHA4* 217/418 (51.9%). Figure 6 shows that the epidermal fluorescence due to hybridization with *LHA1* and *LHA4* gene probes was significantly lower if arbuscules were present in the sections (χ^2 analysis $P\leq 0.01$). Intensity of signal due to *LHA2* gene probe was not significantly altered by the presence of arbuscules ($P\leq 0.213$).

Discussion

We have characterized expression patterns of the three most abundantly expressed P-type H^+-ATPase genes in tomato in whole roots (RNA gel blot hybridization) and at the cellular level (in situ hybridization). Our studies were aimed at characterizing RNA expression patterns of plant H^+-ATPase isoforms within mycorrhizal and non-mycorrhizal roots. From this data, we suggest that specific isoforms may be involved in solute transfer to tomato roots that are in association with arbuscular mycorrhizal fungi.

As the LHA genes have high levels of conservation between them, cross-hybridization between the genes could be problematic. Previous investigations indicated that LHA cDNA sequences do not cross-hybridize under stringent washing conditions (Ewing et al. 1990; Ewing and Bennett 1994); however, these studies used partial sequences (~800–1000 and 209 bp, respectively), and these findings needed to be confirmed with the full-length cDNAs used in the current investigation. Figure 1 indicated that there was virtually no cross-hybridization between the probes, giving confidence that hybridization studies could be used to discriminate the different gene members.

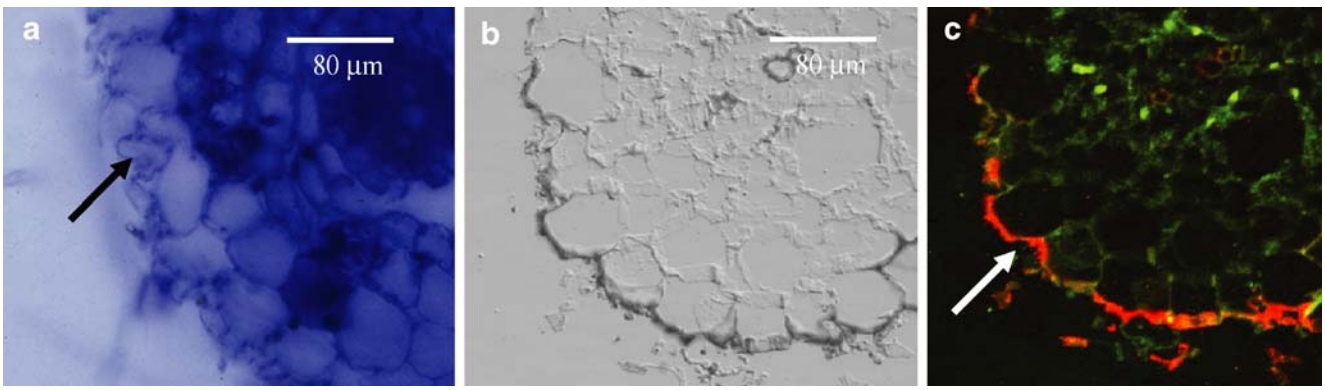

Fig. 3 Transverse sections of *Lycopersicon esculentum* roots. **a** A hand-cut section of a fresh mycorrhizal root stained with trypan blue showing intact epidermal sections (*arrow*). **b** A differential interference contrast (*DIC*) image of a root section that has undergone the in situ hybridization procedure. Epidermal cells have collapsed against the outer cortical cell layer. **c** Section from **b** viewed under laser confocal microscopy. This section was hybridized with the *LHA1* antisense probe, showing expression of *LHA1* in the collapsed epidermal cells (*arrow*)

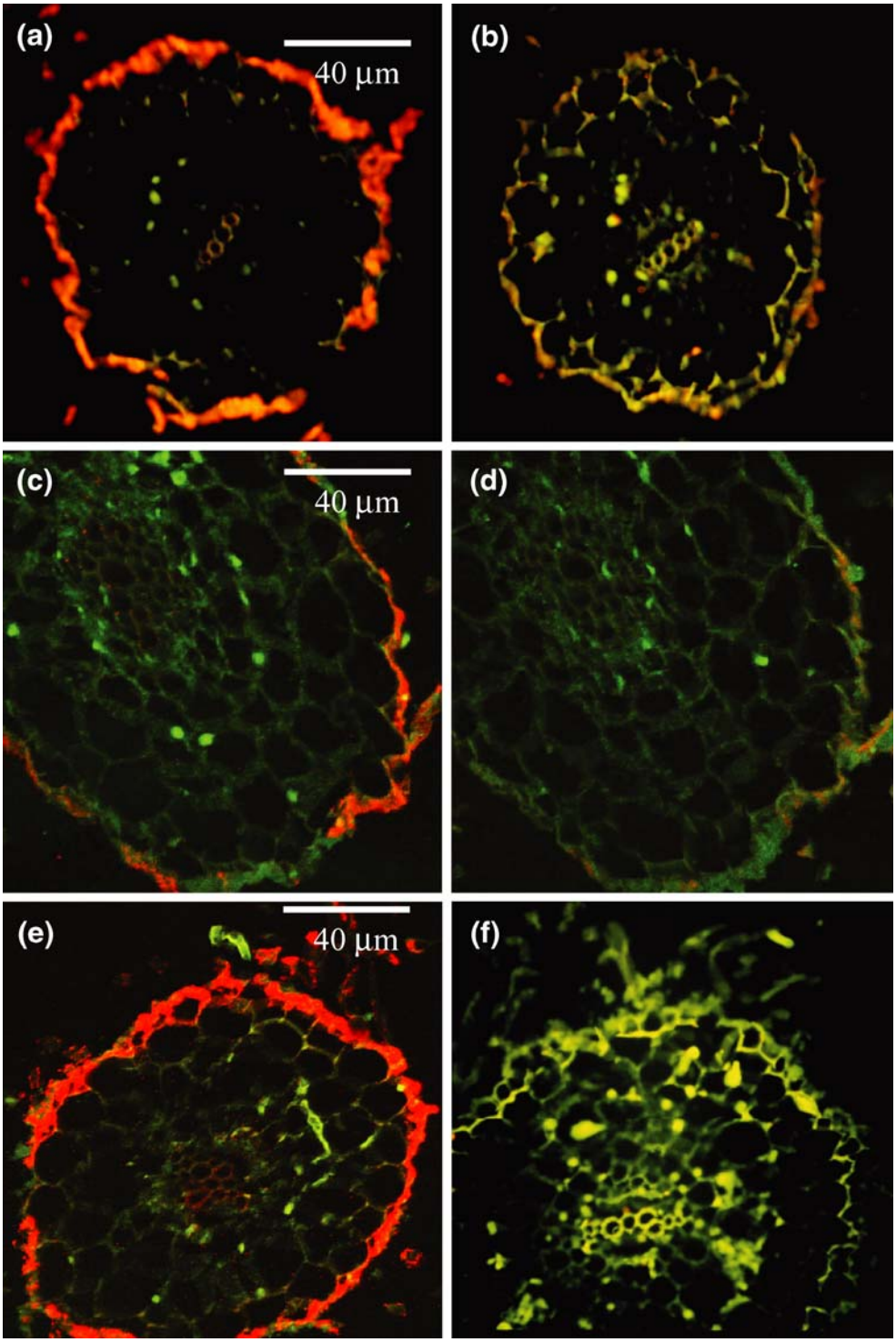

Fig. 4 Laser scanning confocal microscopy images of *Lycopersicon esculentum* roots that have been hybridized with labeled probes from *LHA1* (**a**, **b**), *LHA2* (**c**, **d**), and *LHA4* (**e**, **f**). Antisense probes (**a**, **c**, **e**) and sense probes (**b**, **d**, **f**) were used. Sections comparing antisense and sense signals from the individual genes were taken from within 50–100 um of each other. The HNPP-fast red dye (*orange-red fluorescence*) indicates hybridization to RNA transcripts within cell types

The RNA gel blot analysis investigated gene expression at the whole root organ level. *LHA1* showed significantly higher levels of expression in mycorrhizal roots as compared to non-mycorrhizal roots at 4 DAT. Recent work has indicated that H^+-ATPases are associated with membrane depolarization during the very early stages of a hypersensitive response in Arabidopsis that precedes any visible reaction (Pike et al. 2006). As mycorrhizal fungi have been shown to elicit a small, transient induction of defense related transcripts during appressorium formation (Bonanomi et al. 2001; Blilou et al. 2000; Ruiz-Lozano et al. 1999), it is possible that *LHA1* is similarly involved in such a response at an early stage of mycorrhizal colonization.

Both *LHA1* and *LHA4* expression substantially decreased in mycorrhizal roots between 4 and 10 DAT,

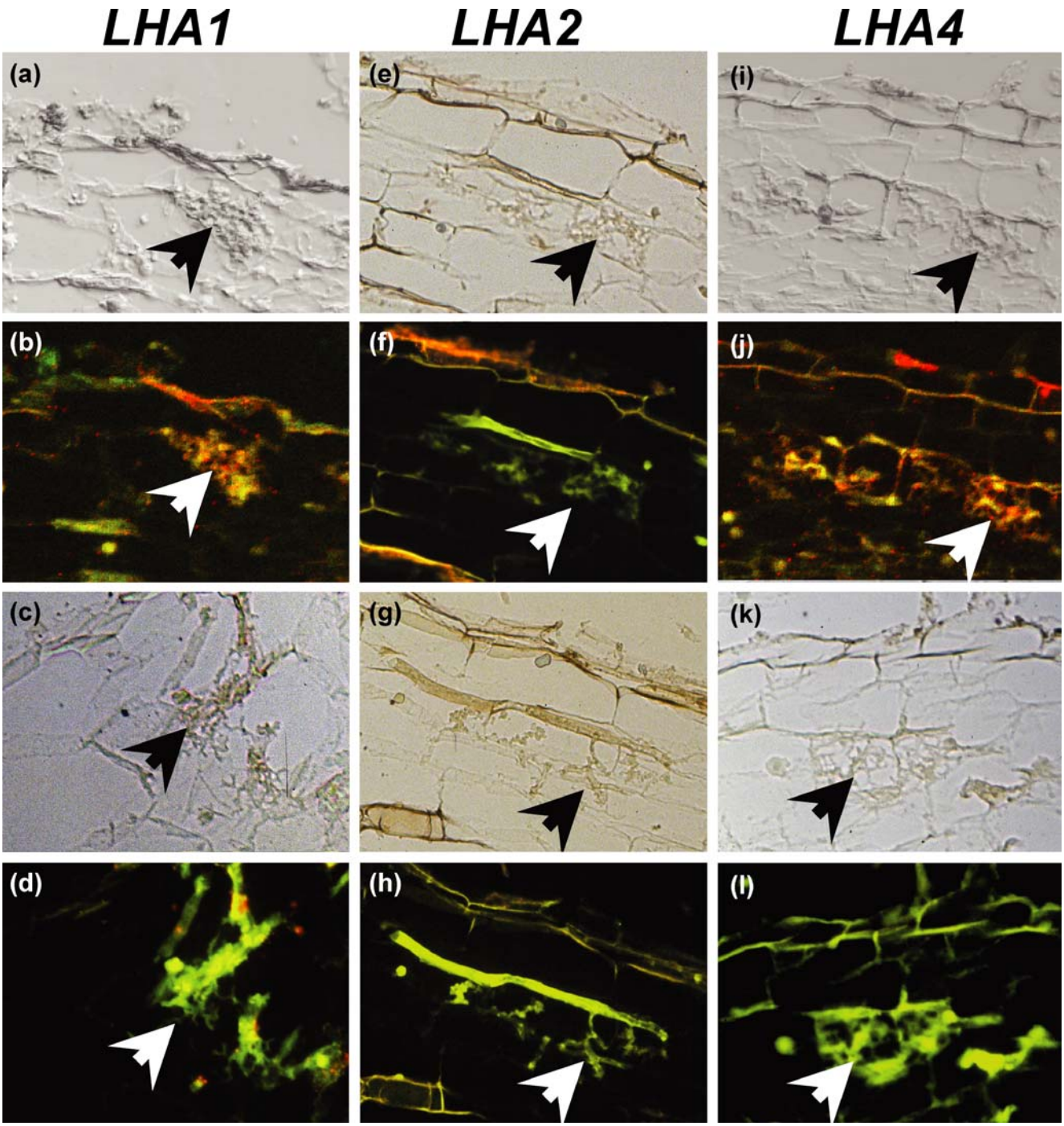

Fig. 5 Hybridization to sections with arbuscule-containing cortical cells using *LHA1* (**a**–**d**), *LHA2* (**e**–**h**) and *LHA4* (**i**–**l**). DIC images (**a**, **c**, **e**, **g**, **i**, **k**) are followed by their respective fluorescent images (**b**, **d**, **f**, **h**, **j**, **l**). Antisense (**b**, **f**, **j**) and sense (**d**, **h**, **l**) hybridizations for each gene are shown. Arbuscules are indicated by *arrows*

whereas *LHA2* expression did not change. These results are in partial agreement with a previous study on ATPase expression in mycorrhizal tomato roots in which reverse transcriptase-polymerase chain reaction with gene-specific primers was used (Ferrol et al. 2002). In both that study and ours, *LHA1* had lower levels of expression in mycorrhizal roots. However, we found different expression patterns for *LHA2* and *LHA4*. Ferrol et al. (2002) observed *LHA2* expression to increase in mycorrhizal roots, while *LHA4* expression remained the same; whereas, we observed *LHA2* expression to remain unchanged, while *LHA4* expression significantly decreased. This could be explained by the use of different fungi, with *G. intraradices* used in this study compared to *G. mosseae* in the Ferrol study. Differences in both function and gene expression between symbioses formed by different species of arbuscular mycorrhizal fungi

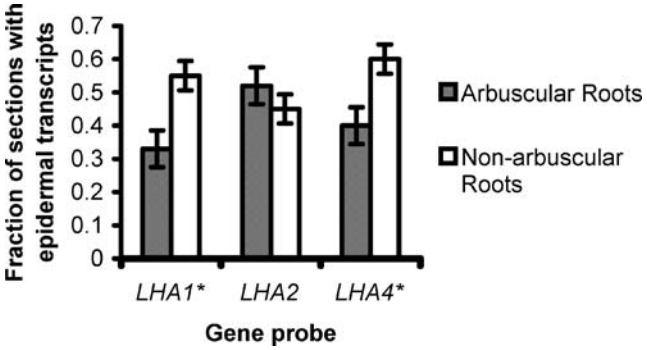

Fig. 6 The proportion of sections (either with or without arbuscules) that showed abundant localization of H^+-ATPase transcripts (*LHA1*, *2*, and *4*) in the epidermis. Means and binomial interval estimation (Agresti and Coull 1998) are shown. *Asterisk* represents significant difference between means at $P<0.01$

are increasingly being identified (e.g., Gao et al. 2004; Smith et al. 2004; Hohnjec et al. 2005). Transcriptional profiling of roots colonized separately with *G. mosseae* and *G. intraradices* not only revealed 201 plant genes being significantly coinduced by both of the fungi, but a further 453 plant genes were induced specifically by *G. mosseae* and 556 specifically induced by *G. intraradices* (Hohnjec et al. 2005). Interestingly, this study showed how two plant phosphate transporters from *Medicago truncaula* (*MtPT1* and *MtPT2*) have different expression patterns when *M. truncatula* was colonized by either *G. mossease* or *G. intraradices*. Similarly, Pozo et al. (1999) also utilized these two fungi and found differential accumulation of glucanases in tomato. It now seems likely that these two fungi elicit very different expression patterns from the same host plant, and this would explain how our observations of *LHA2* and *LHA4* varied from that of Ferrol et al. (2002).

All three ATPase isoforms were localized by in situ hybridization to the outer epidermal cell layer of the plant root. During processing of sections for in situ hybridization, this outer epidermal layer appears to have collapsed in the majority of sections (Fig. 3a and b), although Fig. 4e and f shows well-preserved epidermal cells. However, the collapse of these cells did not interfere with the hybridization process, as we clearly saw high levels of hybridization to the region where the collapsed epidermal cells were located (Fig. 3c). The antisense probes of all three ATPase isoforms generated signals in the epidermal layer of mature roots, and this is shown in Fig. 4a,c and e. The control probes (sense strand of the ATPase sequences) did not generate significant signals in the roots (Fig. 4b,d and f). It is likely that the protein products of these genes are generating a PMF across the plasma membrane of plant epidermal cells, presumably to aid in the uptake of nutrients from the soil, although it cannot be ruled out that these genes may also be involved in other cellular processes. ATPase genes have been shown to be regulated by the application of exogenous sugars (Mito et al. 1996) and high salt conditions (Zhang et al. 1999; Kalampanayil and Wimmers 2001), implying their involvement in solute transport processes. It is not surprising that multiple ATPase isoforms are expressed in the same tissue types as similar results have been observed with ATPases from *N. plumbaginifolia* (Oufattole et al. 2000). The expression of multiple ATPase isoforms in the same tissue type may reflect the large number of different physiological functions that these enzymes perform or may be due to some degree of redundancy between isoforms of this important enzyme.

In the mycorrhizal symbiosis, the fungal partner efficiently scavenges scarce nutrients (e.g., P and Zn) from the soil and transfers them to the plant, presumably through arbuscules located in cortical cells of the plant root. *LHA1* and *LHA4* expression in whole mycorrhizal roots decreased as arbuscules developed (Fig. 2), yet expression of these genes was observed in cortical cells containing arbuscules (Fig. 5). This seems counterintuitive; nevertheless, Rosewarne et al. (1999), using an identical inoculation procedure, observed a similar phenomenon with the tomato phosphate transporter, *LePT1*, and developed a model to explain this observation. RNA gel blot hybridizations measure transcript levels in the whole root, whereas in situ hybridization localizes transcripts to specific cell types. The model described by Rosewarne et al. (1999) speculates that as a mycorrhizal plant in low-phosphate soils attains much of its P through a route provided by the fungus (the interface being at the plant/fungal structure, the arbuscule), the plant will then express transport-related proteins at the arbuscular interface. This would alleviate the need to express plant transporters at sites directly in contact with the soil, thereby generally reducing expression throughout the root, whereas specifically increasing expression in cortical cells containing arbuscule where they are likely to work most efficiently.

In Fig. 6, we provide evidence to support this theory in relation to H^+-ATPase expression. We investigated the number of root sections that had high levels of ATPase expression in the epidermis. This was fairly uniform between the three probes (45.3–51.9%). Of those sections that indicated high levels of epidermal ATPase expression, we scored whether those sections also contained arbuscules or not. *LHA2*, which did not show any expression in cortical cells with arbuscules, nor have any change in expression as arbuscules developed, showed no correlation between epidermal expression and absence of arbuscule. However, both *LHA1* and *LHA4* showed a strong correlation between the absence of arbuscules with the presence of strong epidermal expression. This data provides evidence that although *LHA1* and *LHA4* are certainly expressed in the epidermis, this expression is not uniform along the entire length of the root and, in fact, if sections of the root are heavily colonized and contain many arbuscules, then,

the nearby epidermis has reduced expression of these genes. This type of observation is not unique, as non-uniform protein expression along the root length has previously been demonstrated with an epidermally expressed phosphate transporter protein (StPT2; Gordon-Weeks et al. 2003).

Gianinazzi-Pearson et al. (2000), in a reporter-gene study, also found that two H^+-ATPases were expressed in arbuscule-containing cells of *N. plumbaginifolia*. Our work is complementary to this study, as expression of two H^+-ATPases in arbuscule-containing cells from closely related species has now been shown with two different techniques. Both the *N. plumbaginifolia* and *L. esculentum* H^+-ATPases that are expressed in arbuscule-containing cells are also expressed in a variety of tissue types. Krajinski et al. (2002), on the other hand, showed induction of a specific H^+-ATPase isoform in *Medicago truncatula*, *Mtha1*, in arbuscule-containing cells. Interestingly, *Mtha1* falls into a phylogenetic group different from the *N. plumbaginifolia* or tomato H^+-ATPases that have arbuscule-related expression (Krajinski et al. 2002). With the continued advances in this field, it will be interesting to see if other mycorrhizal induced H^+-ATPases can be identified in *N. plumbaginifolia* or tomato.

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