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Analysis of expression of aquaporins and Na^+/H^+ transporters in tomato colonized by arbuscular mycorrhizal fungi and affected by salt stress

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Abstract

Among the proteins functioning in salt tolerance of plants, Na^+/H^+ transporters and aquaporins appear to be of paramount importance. The present study compares expression of Na^+/H^+ transporter and aquaporin genes in tomato colonized by arbuscular mycorrhizal fungi (AMF) and in non-colonized controls under NaCl stress. As revealed by Northern analyses and in situ hybridizations, expression of two Na^+/H^+ transporter genes is not significantly affected by salt stress or by colonization of the plants with AMF. In contrast, transcript levels of both a tonoplast and a plasmalemma aquaporin gene are reduced by salt stress, and this effect is distinctly enhanced by colonization of the tomato roots with AMF. In leaves, colonization of tomato by AMF results in a drastic increase of the mRNA of all three aquaporin genes assayed under salt stress. Aquaporins are known to significantly contribute to water movement in plants. The results presented here indicate that AMF controls aquaporin expression and thereby presumably regulates water flow in tomato under salt stress.

Keywords: Salt stress; Aquaporins; Na^+/H^+ transporters; Arbuscular mycorrhizal fungi; Salt tolerant plants

1. Introduction

Under diverse stress conditions, most higher plants are colonized by AMF which can have manifold beneficial effects on plant growth (Smith and Read, 1997). Regarding NaCl stress, any alleviation on crop plants via AMF would be of particular interest due to the enormous potentialities in applications. Literature on interactions of salt on arbuscular mycorrhizal fungi (AMF) colonization of plants is somewhat controversial. Reviews (Smith and Read, 1997; Juniper and Abbott, 1993) state that salt inhibits germination of spores or other fungal propagules, colonization of the plant roots and sporulation of AMF. On the other hand, it is known since a long time that plants of salt marshes like the salt aster, *Aster tripolium* (Boullard, 1959), are strongly colonized by AMF. Several publications report that AMF in saline soils can decrease plant yield losses by increasing their salt tolerances (Cantrell and Linderman, 2001; Al-Karaki et al., 2001; Hatimi, 1999; Ruiz-Lozano et al., 1996; Tsang and Maun, 1999). The mechanisms underlying the salt tolerance conferred by AMF have not yet been elucidated at a molecular level.

Plant salt tolerance itself is a complex trait (Shi et al., 2000) to which many different factors may contribute: generation of osmoprotectants (proline, glycinebetaine, polyols) in the cytoplasm, energy supply by ATPases for the export of Na^+ and Cl^-, specific transport proteins for the transfer of these ions into the vacuole or into the apoplastic spaces, additional water supply mediated by aquaporins to maintain osmobalance and others (Hasegawa et al., 2000). Furthermore, any comparative study in the field is faced with the problem that such factors are generally encoded by gene families. For Na^+/H^+ antiporters, e.g., 6 fully sequenced members and at least 40 potential other candidates have been recognized already 3 years ago (Xia et al., 2002). The genome of *Arabidopsis* encodes 35 aquaporin genes with 13 homologs in the plasma membrane intrinsic protein (PIP) subgroup (Jang et al., 2004). Thus, any study on the impact of mycorrhizal colonization on the expression of genes with products involved

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in salt tolerance is faced with the multiplicity and complexity of the traits. In gene expression studies, one can concentrate only on few ones which are seemingly of paramount relevance in salt tolerance. The current research on salt tolerance focuses on: (a) aquaporins and (b) Na^+/H^+ antiporters.

(a) The Na^+/H^+ antiporters catalyze the transfer of Na^+ out of the cytoplasm into either vacuole or apoplasm. Transgenic plants with overexpressed Na^+/H^+ antiporters were reported to be more salt tolerant than the controls as shown for *Arabidopsis* (Gaxiola et al., 1999; Sottosanto et al., 2004) or rice (Fukuda et al., 1999).
(b) Due to the very negative water potential in saline habitats, plants must maintain their osmotic balance in the cytoplasm, and are faced with the problem to acquire sufficient amount of water. Indeed, even early investigators recognized that plants in saline soils have to cope with drought problems and that glycophytes rapidly wilt under salt stress (Stocker, 1928). The role of the aquaporins in plants has not been fully explored. However, it is believed that water passes to a large extent through the plasmalemma or the tonoplast through channels formed by aquaporins (Maurel, 1997; Zeuthen, 2001; Hill et al., 2004). A correlation could well exist between the expression or activity of aquaporins and salt sensitivity of plants (Johansson et al., 2000).

The present study is seemingly the first attempt to elucidate the effects of AMF in salt stressed plants on the molecular level. The expressions of aquaporins and Na^+/H^+ antiporter genes in AMF colonized and control tomato plants, treated and non-treated by sodium chloride, were studied by Northern analysis and in situ hybridization. The results will show that the expression of the genes studied is differentially affected by salt treatment and AMF colonization.

2. Materials and methods

2.1. Organisms used and their growth conditions

Tomato (*Lycopersicon esculentum* Mill. var. Tamina) seeds (purchased from Schmitz and Laux, D-Hilden) were surface-sterilized by treatment with 70% ethanol (5 min) and then with 0.1% $HgCl_2$ (5 min) followed by washing with sterilized distilled water (5 min, 5×).

For the NaCl treatment, plants (and fungi) were grown in 1 L pots containing a mixture of 10% sterilized soil ("Einheitserde", from Steuder, D-Schermbeck), 15% Lecaton (Ø 2–4 mm, pore width 75%) with the fungal spores and propagules and 75% sterilized quartz sand. The first 6 weeks of growth, the pots were not supplemented with NaCl. Plants were watered daily, once a week with a 1:1 diluted Hoagland solution but without phosphorus and once a month with Hoagland solution including phosphorus to ensure a high degree of mycorrhizal colonization of the roots. In the 7th week, the plants were watered with a $NaCl/H_2O$ solution to reach a final concentration of 0.1% in the pot mixture. This concentration was increased to 0.2% the next week, to 0.4% the 9th week and then finally to 0.8% the 10th week. The concentration was then kept to 0.8% until the 17th week when the plants were harvested by transfer to liquid nitrogen and stored at −70 °C prior to the assays. The concentration of NaCl in the growth substrate (=pots) was regularly monitored by measuring the electric conductivity (EC) using the sensor IMAG-DLO of the Institute of Agricultural and Environmental Engineering, Wageningen, The Netherlands. For converting EC to % NaCl, the formula of Richards (1954) was used. The NaCl/water solution was adjusted accordingly when the concentration in the pot substrate was lower than 0.8%.

The fungal inoculum consisted of a mixture of *Glomus geosporum* and *Glomus intraradices* to approximately the same part in Lecaton (from Leca, D-Halstenbek). *G. intraradices* (Schenk & Smith INVAM 167) was originally obtained from the late Professor H. Marschner, Stuttgart-Hohenheim and maintained in our laboratory since years. *G. geosporum* was isolated from roots of the salt aster, *A. tripolium*, of the salt marsh at D-Jerxheim (Landwehr et al., 2002) and grown up to inoculum production using *Tagetes* as host plant.

Mycorrhizal colonization was determined by a modified version of the gridline intersect method (Schmitz et al., 1991).

2.2. Isolation of DNA, RNA and Northern blot analyses

DNA from roots and leaves was isolated as described by Raeder and Broda (1985) and RNA as by Eggermont et al. (1996). For the Northern analyses, RNA (20 μg/lane) was separated on 1.2% formaldehyde agarose gels. Northern hybridizations were performed with digoxigenin-labelled RNA probes. The vector pGEM-Teasy, containing the cloned PCR products, was linearized and used as template for the in vitro transcription with Sp6 or T7 RNA polymerase yielding the dixoxigenin labelled antisense probes (also the sense probes for the in situ hybridizations). The signal intensities of the Northern blots were quantified by using the NIH Image 1.62 Computer Programme. To have calibrations of the signal intensities, the filters were subsequently stripped and hybridized with an 18S-rDNA gene probe. This was obtained by amplifying the nucleotides 1575–1583 (=X51576) of tomato RNA by PCR. The lowest signal intensity in one lane among all of one blot was set to 1.0, and the signal strength of the others was thus compared among each other. Corrections in the calibration were performed if the amount of 18S rRNA loaded was not the same for each lane.

The Superscript Preamplification System for First Strand Synthesis (Invitrogen, D-Karlsruhe) was used for the synthesis of cDNA from total RNA (2 μg/reaction) of tomato roots or leaves. Contaminant DNA was removed by incubation with DNaseI (amplification grade, Invitrogen). The RNA obtained was denatured by incubation at 65 °C (10 min).

Reverse transcription was started by using the RACE 1 oligo dT-primer (see legend of Table 2). The cDNA obtained was digested with RNase H (2 U/μL, from Invitrogen) and purified with the QIAquick PCR Purification Kit (Qiagen, D-Hilden).

2.3. PCR protocol to amplify segments of aquaporin and Na^+/H^+ transporter genes

Using DNA from tomato roots as template, one segment of each the PIP and TIP gene was amplified by PCR. Primer sequences are given in Table 2. RACE-PCR (Frohman et al., 1988) with cDNA from tomato roots provided segments containing the 3′-non-translated region of members of the PIP and TIP aquaporin gene families. Together with the primers RACE1, RACE2, RACE3, the gene specific primers PMA and PM1 yielded *LePIP1* (AY725511, new PCR-product), PMA and PM2 *LePIP2* (BT014251) and TPA and TP1 *LeTIP* (AY731066, also new PCR-product).

In the case of the Na^+/H^+ transporter genes, the primer pair ISO1/ISO11 was used with tomato root cDNA as a template for amplifying *LeNHX1* (AJ306630) and ISO2/ISO22 for *LeNHX2* (AJ306631).

The obtained PCR products were cloned into the pGEM-Teasy vector (Promega, Madison, USA) and sequenced. Their homologies to deposits are given in Table 2. The cloned PCR products were then used for the production of DIG-labelled riboprobes.

2.4. Quantification of the mRNA by real-time PCR

This was performed for the tonoplast aquaporin *LeTIP* in a GeneAmp 5700 Sequence Detector System (Applied Biosystems, D-Weiterstadt) with the following thermal programme: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 92 °C, 1 min at 60 °C using AmpliTaq Gold DNA polymerase. Fluorescence of the amplificates was detected with the SYBR Green PCR Master Mix (Applied Biosystems) and quantified using the GenAmp 5700 sequence detection system software. The primers in real-time PCR were TRP1: TCA TTC GTG GAG GAT TGG CT and TRP2: ACA CTG TTG AAG ACT TGT TCT TCT TTC TTC T. Standardization for this amplificate was performed against the 18S-rDNA using the primers 18S-1: GAT GCG CTC CTG GCC TTA AT and 18S-2: TAC AGA GCG TAG GCT TGC TTT.

2.5. In situ hybridization experiments

This method, described earlier in detail (Kaldorf et al., 1999), was only slightly modified for the current study. Roots were fixed in 4% formaldehyde for 16 h at 4 °C, infiltrated with xylene and embedded in Histowax (Leica). Longitudinal sections (12 μm) were mounted on (3-aminopropyl)-trimethoxysilane-coated slides. The sections were treated with 0.125 mg/mL pronase and fixed with 4% formaldehyde. Digoxigenin-labelled sense and antisense RNA probes were obtained by in vitro transcription of linearized vectors containing the cloned PCR-products. To obtain these PCR-products, the following primers were used for *PIP1*F: GAG AGC TGC AGC TAT TAA GGC, for *PIP1*R: TAA CCA ATA CTAATA AGG ATG (length of the PCR-product obtained 224 bp), for *TIP*F: GTC GGA CCA CTC ATT GGT GGA and for *TIP*R: GAA CCA AACATG ATA TTC CAT (236 bp). The hybridization solution (50% formamide, 20 μg/μL *t*-RNA, 30 mM NaCl, 1× Denhardt's solution, 10% dextran sulfate) was added to the probes which had a final concentration of 2 ng/μL). Hybridization was performed for 16 h at 50 °C. Signals were detected with anti-digoxigenin-antibodies coupled to alkaline phosphatase. Photographing was done with a Hamamatsu digital camera C4742-95.

3. Results

3.1. Characterization of the tomato plants used for the experiments

Mycorrhizal and non-mycorrhizal tomato plants were subjected to salt stress after the 6th week of growth. To avoid a salt shock, the NaCl concentration was increased stepwise up to 0.8%, and this concentration was kept constant till the 17th week (see Section 2). At harvest after 17 weeks of growth, the fresh weight of the plants in total was not significantly different between AMF colonized tomato and the controls both in non-stressed (no NaCl added) plants and in pots containing 8 g/L NaCl (Table 1). Likewise, the fresh weight of roots or shoots was not different between AMF- and non-colonized tomato in the non-treated plants. However, treatment with 8 g/L NaCl reduced the fresh weight of roots to approximately 50% of the value obtained with non-treated plants in the AMF colonized material and this change was statistically significant (Table 1). The shoot weight appeared to be slightly increased (however, the difference was not statistically sound). Roots and shoots of AMF colonized plants had the same fresh weight as the non-colonized controls when treated with 8 g/L NaCl (Table 1). The same result was obtained in three independent growth experiments. The degree of mycorrhizal colonization was $74 \pm 8.9\%$ ($n = 5$) in the non-salt-treated plants, whereas it was $84 \pm 6.0\%$ ($n = 5$) in the plants supplemented with 8 g/L NaCl. Controls which had not been inoculated with AMF showed no mycorrhizal colonization of the roots. Plants containing 1.2% NaCl in the growth substrate did not survive within the 17 weeks.

3.2. Development of gene probes for aquaporins and Na^+/H^+ transporters

Using the primer pair PMA/PMB, genomic DNA provided two PCR-products that were cloned and sequenced. One PCR product of 500 bp length showed 100% sequence homology in two areas of 285 bp (85%) or 128 bp (90%), respectively,

Table 1
Fresh weight of the tomato plants used for the gene expression analysis

Treatment	Growth condition	Fresh weight of		
		Total plants	Roots	Shoots + leaves
With 0% NaCl	Mycorrhizal	53.8 ± 14.6	13.6 ± 6.1	40.2 ± 8.5
	Non-mycorrhizal control	61.3 ± 6.9	16.5 ± 3.0	44.8 ± 5.1
With 0.8% NaCl	Mycorrhizal	54.5 ± 6.6	8.4 ± 0.5	46.1 ± 6.7
	Non-mycorrhizal control	55.6 ± 7.3	9.6 ± 1.5	46.0 ± 7.4

The fresh weight of the plants was determined after the 17th week of growth including 8 weeks treatment with 0.8% NaCl from the 10th week on (except for the controls; for details, see Section 2). Standard deviations for each determination ($n = 5$ plants in each case).

to a plasma membrane aquaporin (=PIP) sequence from *Nicotiana tabacum* (AF440271) and apparently contained an intron of 87 bp. The other one of 794 bp had a homology on 237 bp (91%) or 141 bp (90%) to a PIP sequence of *Solanum tuberosum* (Y18311) and had two putative introns of 283 and 133 bp, respectively. Surprisingly the tomato PIP sequence (AF218774), which had been used to design the primer pair PMA/PMB, was not obtained in this assay. The sequence information of the two products was used to synthesize two new gene specific primers (PM1 and PM2) that were specifically applied in a 3′-RACE approach to obtain gene segments containing the 3′-non-translated regions of the respective genes (Table 2). The PCR-product obtained with PM1 (*LePIP1*, 722 bp, accession number AY725511) was 100% identical to a tomato EST (CK715300, corresponding to the *PIP2* type of Schäffner, 1998), whereas the other one obtained with PM2 (*LePIP2*, 527 bp) was identical to a corresponding mRNA sequence from tomato (BT014251, thus the *PIP1* type of Schäffner, 1998). Since the 3′ends of genes are non-conserved and non-translated, the obtained gene probes *LePIP1* and *LePIP2* were only to 65% identical, at the DNA level, and were thus specific enough to discriminate in their gene expression.

Similarly, a 300 bp PCR-product was amplified from genomic DNA with the primers TPA and TPB for a tonoplast aquaporin (AY731066). The subsequent 3′-RACE provided a 606 bp gene segment (*LeTIP*) with 100% identity (437 bp) to a sequence deposited for tomato (U95008, corresponding to δ-*TIP* type of Zardoya and Villalba, 2001) using the primer TP1 (Table 2).

The primer pairs ISO1/ISO11 and ISO2/ISO22 allowed to amplify 692 bp segments of the tomato Na^+/H^+-antiporter genes *LeNHX1* (AJ306630) and *LeNHX2* (AJ306631), respectively. Both sequences were 100% identical to the published sequence for these isoforms (Venema et al., 2003, see also Table 2). Among each other, the two sequences showed 40% similarity, at the DNA level, and were thus different enough to forbid cross-hybridizations.

3.3. Expression studies of the LeTIP, LePIP and Na^+/H^+ antiporter genes (Northern analyses)

For these analyses (Fig. 1), RNA was isolated from roots and leaves of both the NaCl-stressed and the non-NaCl-treated control plants to assess any differential expression of the genes mentioned. In roots, the transcript level

Table 2
Gene probes developed for the present study

Enzyme	Name of the probe developed	Length (bp)	Primer pair used and	Accession number	Sequence homology to a segment deposited
Plasma membrane aquaporin	*LePIP1*		PMA/PMB		80% to AY08150 of *Arabidopsis* (483 bp)
		722	PMA/PM1/RACE1, 2, 3	AY725511[a]	
	LePIP2		PMA/PMB		100% (527 bp)
		527	PMA/PM2/RACE1, 2, 3	BT014251	
Tonoplast aquaporin	*LeTIP*		TPA/TPB		100% (437 bp)
		606	TPA/TP1/RACE1, 2, 3	AY731066[a]	
Na^+/H^+ antiporter	*LeNHX1*		NAH1/NAH2		
		692	ISO1/ISO11	AJ306630	100% (692 bp)
	LeNHX2	692	ISO2/ISO22	CAC8306	100% (692 bp)

Sequences of the primers used. PMA: CACATTAACCCTGCTGTGACA; PMB: AGGTCCCACCCAGAAAATCCA; PM1: GGCAAGAAAAGTATCACTAATCA, PM2: TGGTGGTGCTAATGTTGTTA; TPA: GGTGGACATTTGAATCCAGCTGT: TPB: ATGTTGGCACCAACAATAAA; TP1: GGGTTGCTCAATTACTTGG; ISO1: TCCTGGAAAATCTGTTGGGG; ISO11: TGAATCCCCTTTAGCTCCGC; ISO2: ATTTATCTTCTTCTCAATTC; ISO22: CTTACAATCAACAAGCCACT; RACE1: CCACGAGTCGACTCTAGAGCTCGGATCCTTTTTTTTTTTTTTTT; RACE2: CCACGAGTCGACTCTAG; RACE3: CTCTAGAGCTCGGATCC.

[a] Own deposit.

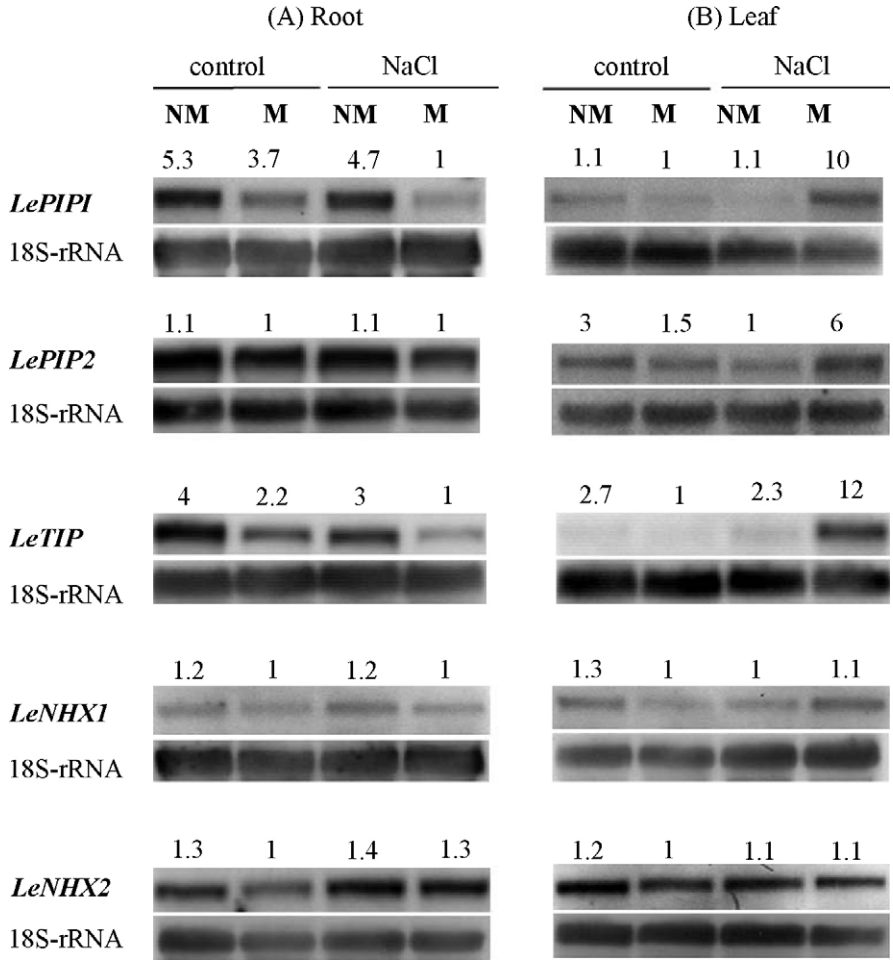

Fig. 1. Northern hybridizations using total mRNA and the different digoxigenin-labelled riboprobes for aquaporins and Na^+/H^+ antiporters. Lowest signal intensity of one lane was arbitrarily set to 1 to allow a comparison of the signal strengths within one blot (bloc of four lanes) for each gene expressed. Signal strengths were adjusted to the amount of 18S rRNA blotted onto each lane. (A) Root and (B) leaf. M: mycorrhizal plants, NM: non-mycorrhizal plants, control: plants not stressed with NaCl.

of the aquaporins *LePIP1* and *LeTIP* was highest in non-mycorrhizal plants, regardless of being salt-treated or not, and mycorrhizal colonization drastically decreased the transcript amount in roots (Fig. 1A). Salt stress decreased the amounts of transcripts of *LePIP1* and *LeTIP*, both in leaves and roots of either mycorrhizal or non-mycorrhizal plants, but the effect caused by NaCl was not so distinct as the one by mycorrhizal colonization. In contrast, the Northern bands showed that *LePIP2* expression was virtually unaffected by all treatments. In leaves, however, all three genes were strongly expressed in mycorrhizal plants upon salt stress (Fig. 1B). Differences were even in the range of one order of magnitude compared to the expression in non-mycorrhizal plants and to non-salt-treated, control tomato in two independent experiments with different plant material. The expression of the Na^+/H^+ antiporter genes, however, was not significantly affected by mycorrhizal colonization and salt stress, in both roots (Fig. 1A) and leaves (Fig. 1B). Under the growth conditions assayed, *LeNHX2* was distinctly expressed whereas the Northern bands for *LeNHX1* were only weak both in roots and leaves.

To check the validity of the results, the data for one gene (*LeTIP*) were verified by quantitative real-time PCR and thus by an independent method (Fig. 2). Mycorrhizal colonization reduced the amount of transcripts significantly in roots of both salt-treated plants and in the controls, whereas as the level of transcripts was by far highest in leaves of mycorrhizal plants under NaCl stress.

3.4. In situ hybridization experiments

To assess the spatial expression of transcripts in *roots*, in situ hybridization experiments were performed with *LePIP1* and *LeTIP* (Fig. 3). These assays were performed with salt-treated plants, on which the Northern analysis data had suggested that the differences in transcripts between mycorrhizal and non-mycorrhizal plants were so high that differences in the in situ images could be expected. In the case of *LePIP1*,

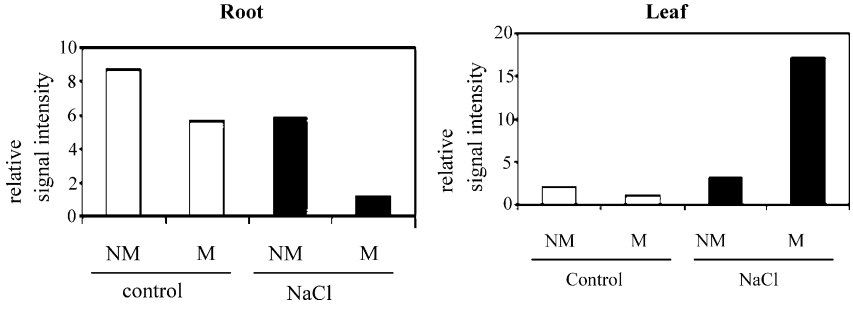

Fig. 2. Real-time PCR with the tonoplast aquaporin *LeTIP* with cDNA from roots and leaves of tomato. For experimental details, see Section 2. Black bars: plants treated with 0.8% NaCl. White bars: non-salt stressed plants. M: mycorrhizal, NM: non-mycorrhizal plants.

the root parenchyma cells of non-colonized plants showed distinct signals (blue colour), and the colour intensity was highest next to the endodermis cells with the antisense probes (Fig. 3A and B). The vascular tissue was essentially free of signal formation. The same pattern was detected when side roots were cut and stained. *LePIP1* was apparently expressed only (or mainly) in the root parenchyma cells till the endodermis. The sense control (Fig. 3C and F) provided faint, unspecific signals. In mycorrhizal roots, the visible signals of *LePIP1* were distinctly lower than in non-mycorrhizal controls, and once more, signals were detected in the inner root parenchyma cells along the endodermis (Fig. 3D and E). The signal (blue colour) was even stronger for *LeTIP* (Fig. 3K and L) than for *LePIP1*, and transcript formation was more or less evenly distributed within the roots, thus also in the vascular tissue. Mycorrhizal colonization reduced the transcript amount (intensity of the colour) significantly (Fig. 3N and O). Sense controls (Fig. 3P) were only faintly coloured, and the blue colour centred around arbuscules in colonized roots (similar as with the antisense probes), thus unexpectedly corroborating that the plants were, indeed, strongly AMF colonized.

Cross-sections of *leaves* of salt-treated tomato plants corroborated for *LePIP1* that signal intensity was higher in AMF colonized plants (Fig. 3G) than in the controls (Fig. 3I). A comparison of the signal intensities obtained with the antisense and sense probe revealed that *LePIP1* was expressed mainly in the outer epiderminal cells and in the tissue embedding the steles in leaves of AMF colonized tomato (Fig. 3G). In leaves of non-colonized plants, only faint signals were detected, however, in the same tissues as with the AMF-colonized leaf material (Fig. 3I).

4. Discussion

The major outcome of the present study was that the expression of aquaporins but not of the Na^+/H^+ antiporters studied is strongly impaired by mycorrhizal colonization and salt load. Aquaporins belong to the major intrinsic protein (MIP) family of transmembrane channels, which permit selective membrane passage of water (and few other compounds) but not of H^+ and other ions (Weig et al., 1997; Chen et al., 2001; Hill et al., 2004) through the plasmalemma (by PIPs) and the tonoplast (by TIPs). Aquaporins are regulated both at transcriptional and activity levels. Aquaporin activity is controlled by phosphorylation (Johansson et al., 1996; Maurel et al., 1995), which appears to be a short-term response to stresses like drought and salinity. In the longer run, water transport by aquaporins can be down-regulated by the reduction of transcript formation. This is in accordance with earlier biophysical measurements, which showed that salinity has adverse effects on water transport (Azaizeh and Steudle, 1991; Azaizeh et al., 1992). NaCl decreases the osmotic pressure dependent hydraulic conductance in roots (Carvajal et al., 1999). The correlation between water permeability in roots and expression of specific aquaporins has not been proven but seems likely (Kaldenhoff et al., 1998; Bastías et al., 2004; Aroca et al., 2005). In ice plants, transcript levels of some aquaporins are down-regulated in the first 30 h after exposure to salt stress and recover when this stress is interrupted (Yamada et al., 1995). Likewise, NaCl reduces either the activity or abundance of Hg-sensitive water channels (aquaporins) as shown by measuring the osmotic pressure-dependent hydraulic conductivity of detached, exuding root systems from paprika pepper (Carvajal et al., 1999) and from melon (Carvajal et al., 2000). In line with these observations, the amount of *LeTIP* and *LePIP1* but not of *LePIP2* transcripts was found to be down-regulated by roughly 20% under continuous salt stress in the present communication (Fig. 1). The fresh weight of roots (but not of shoots) was impaired by the salt treatment. Thus, roots seemingly osmoregulate their solutes in roots by reducing water entry into cells and by forming less cell material. It must, however, be mentioned that other authors report an up-regulation of aquaporin mRNA transcripts under salt stress, e.g., in *Nicotiana excelsior* (Yamada et al., 1997), *Arabidopsis thaliana* (Gaxiola et al., 1999), *Oryza sativa* (Fukuda et al., 1999) and *Beta vulgaris* (Xia et al., 2002). Such differences may be a consequence of the mode of the salt stress set, the differences between plant species tested and the complexity in expression pattern of different members of the large family of aquaporins (Sarda et al., 1999). The effect on transcript formation of aquaporins was more drastic after AMF colonization than after salinity. In the non-treated controls but even more in salt stressed roots, AMF significantly reduce the mRNA

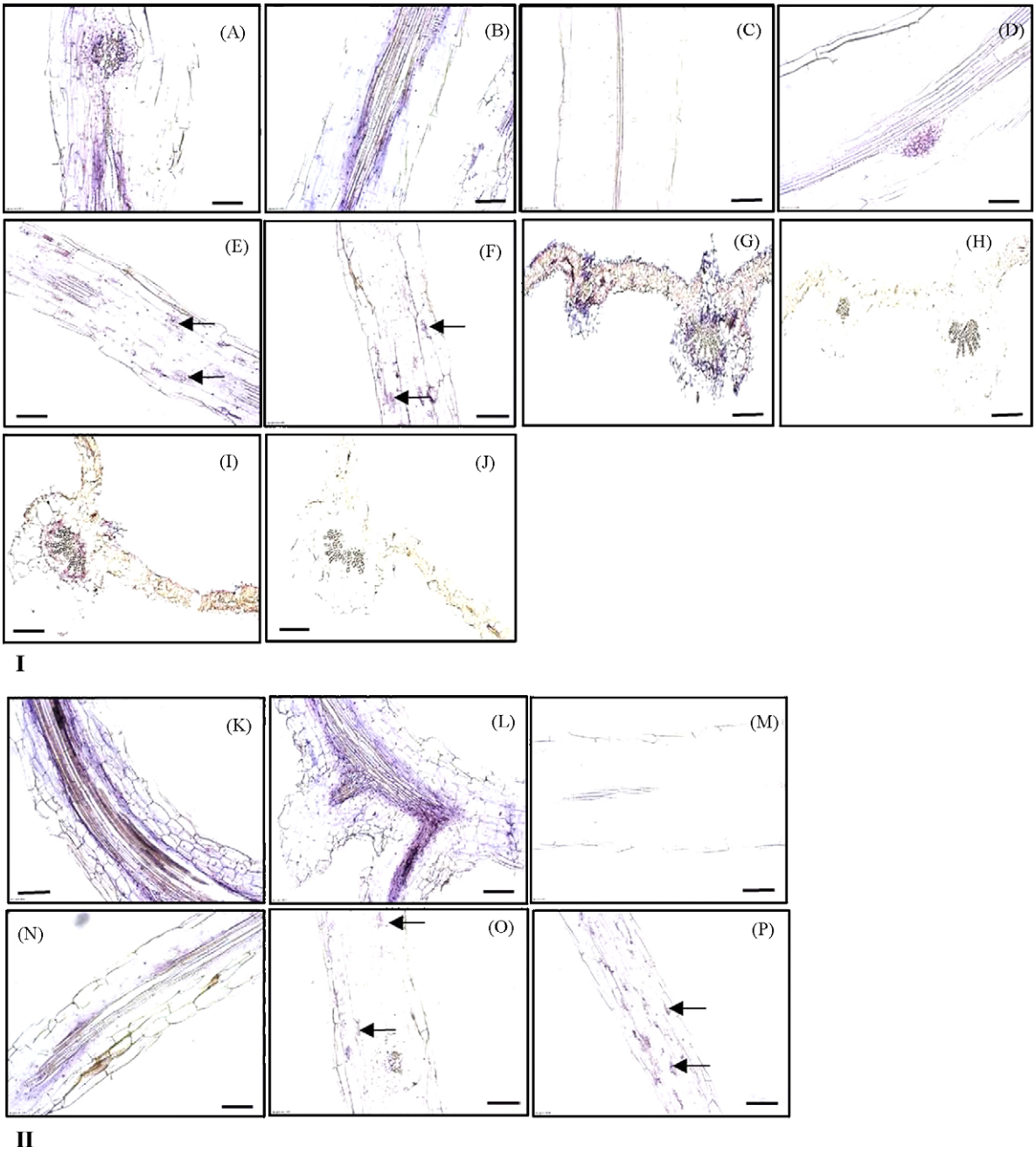

Fig. 3. In situ hybridizations using cross-sections of tomato hybridized with the antisense and sense probes of *LePIP1* and *LeTIP*. Transcripts amounts for *LePIP1* or *LeTIP* are visualized by the intensity of the blue colour developed by the reaction with anti-digoxigenin antibodies coupled to alkaline phosphatase using NBT/BCIP as substrate. Scale of the bar: 100 μm. Arrow indicates an arbuscule. Few arbuscules are only visible due to the thinness of the cut (=12 μm). I: graphs for *LePIP1*; II: graphs for *LeTIP*; A, B, D, E, G, I, K, L, N, O: hybridization with the antisense probe; C, F, H, J, M, P: hybridization with the sense control; D–H and N–P: mycorrhizal plants; A–C and I–M: non-mycorrhizal control plants; A–F and K–P: cross-sections through roots; G–J cross-sections through leaves.

transcripts of *LePIP1* and *LeTIP* but not of *LePIP2*, indicating that AMF differentially exert controls on expression of these genes. Even more strikingly, all signals of all three aquaporins were faint but distinct in leaves and these signals were strongly increased only in leaves after the NaCl treatment in AMF colonized plants. If water transport in plants is, indeed, mediated, to a large extent, by aquaporins, then mycorrhizal fungi have strong impacts on the water supply of plants. It has been shown earlier that colonization of cucumber by AMF enhances water uptake into plants under salt or drought stress (Rosendahl and Rosendahl, 1991; Augé, 2001). If the reduction of transcript formation in roots reflects a reduction in aquaporin activity of tomato cells, then the fungi might effectively take over the mobilization of water

under salinity and also mediate its transfer from roots to the shoots. It is, however, not clear how this is achieved, since fungal structures do not pass the endodermis barrier. The enhanced expression of aquaporin genes in shoots may also reflect an enhanced supply with phosphorus and other minerals in shoots which coincidentally may also demand an improved water supply. It should also be mentioned that, by contrast, an enhanced expression by AMF has been reported for another aquaporin in roots of *Medicago truncatula* (Krajinski et al., 2000).

Overexpression of Na^+/H^+ antiporters in plants or microorganisms renders them more tolerant to salt stress (Zhang et al., 2001; Waditee et al., 2002). Thus, analyzing expression of two Na^+/H^+ antiporter genes in dependence on salt and mycorrhizal colonization was of particular interest in the present study. Under the conditions employed, no significant alterations were detected in the expression of *LeNHX1* and *LeNHX2*, the latter of which has recently been shown to be a K^+/H^+ transporter (Venema et al., 2003). If there were any alterations in the expression of these genes in dependence on salt stress and mycorrhizal colonization they were marginal compared to those seen with aquaporins. Similarly as in the present study, transcript levels of *NHX1* of *Arabidopsis* were also not up-regulated in response to NaCl (Apse et al., 1999). It should, however, be stated that others do report up-regulation of tonoplast or plasmalemma Na^+/H^+ antiporter genes in dependence of salt load, in *N. excelsior* (Yamada et al., 1997), *Arabidopsis* (Gaxiola et al., 1999) or *O. sativa* (Fukuda et al., 1999). The increases reported in these publications appear to be maximally three-fold and are thus not so drastic as with the aquaporins in the present study. As stated (Apse et al., 1999), inducing Na^+/H^+ antiporter synthesis requires unknown conditions, and this situation has not changed now some 5 years after this publication. AMF colonization unlikely triggers the expression or activity of Na^+/H^+ antiporter genes. It is somewhat surprising to us how the overexpression of a vacuolar Na^+/H^+ antiporter can confer salt tolerance to plants. When this gene is overexpressed upon salt stress, dramatic osmotic adjustments are expected to occur and also the counterion chloride must be kept away from the cell cytoplasm.

The present study aimed at finding genes that are differentially expressed in AMF colonized control tomato plants when exposed to salt stress. For this purpose, a mixture of *G. intraradices* and *G. geosporum* was chosen. The *G. intraradices* isolate used, Sy167, is the best plant colonizer under the conditions of our laboratory, but the present study (data not documented) revealed that it is not particularly adapted to salt stress. *G. geosporum* was isolated from a salt marsh (Landwehr et al., 2002; Hildebrandt et al., 2001). Previous studies showed that *G. geosporum* spores abundantly occur in different salt marshes (Hildebrandt et al., 2001). It is thus tempting to assume that *G. geosporum* confers tolerance to plants being strongly colonized in salt marshes. However, our repeated attempts with several plant–fungal combinations under different experimental conditions consistently failed to find a *G. geosporum* or another isolate which confers salt tolerance to plants (unpublished data). There are, however, positive reports from other laboratories (Ruiz-Lozano et al., 1996; Cantrell and Linderman, 2001; Hatimi, 1999; Tsang and Maun, 1999). Salt tolerant AMF isolates have been obtained (Ruiz-Lozano and Azcón, 2000) and also tomato cultivars differ in salt tolerance to inoculation with mycorrhizal fungi under salt stress (Al-Karaki et al., 2001). Differences in the findings of the laboratories may be explained by different experimental conditions employed to assay salt tolerance. No standardized protocol exists to assess salt tolerance and to keep salt concentration in the medium (soil) constant. Salt is easily washed out when plants (soils) are drained. When salt concentration is kept constant in the medium as in the present study, then AMF strongly affect the expression of aquaporins upon salt stress. In addition, AMF have severe impacts on the NaCl allocation within roots, as recently documented by PIXE measurements with roots of the salt aster, *A. tripolium* (Scheloske et al., 2004). Despite our own negative attempts till-now we are still optimistic that an AMF inoculum can be developed which confers salt tolerance to plants under constant high soil salinity with a broad potential for applications.

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