*Mtha*1, a Plasma Membrane H$^+$-ATPase Gene from *Medicago truncatula*, Shows Arbuscule-Specific Induced Expression in Mycorrhizal Tissue

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Abstract: Transport processes between plant and fungal cells are key elements in arbuscular mycorrhiza (AM), where H$^+$-ATPases are considered to be involved in active uptake of nutrients from the symbiotic interface. Genes encoding H$^+$-ATPases were identified in the genome of *Medicago truncatula* and three cDNA fragments of the H$^+$-ATPase gene family (*Mtha*1–3) were obtained by RT-PCR using RNA from *M. truncatula* mycorrhizal roots as template. While *Mtha*2 and *Mtha*3 appeared to be constitutively expressed in roots and unaffected by AM development, transcripts of *Mtha*1 could only be detected in AM tissues and not in controls. Further analyses by RT-PCR revealed that *Mtha*1 transcripts are not detectable in shoots and phosphate availability did not affect RNA accumulation of the gene. Localization of transcripts by *in situ* hybridization on AM tissues showed that *Mtha*1 RNA accumulates only in cells containing fungal arbuscules. This is the first report of arbuscule-specific induced expression of a plant H$^+$-ATPase gene in mycorrhizal tissues.

Key words: Arbuscular mycorrhiza, *in situ* hybridization, plant nutrition, RNA accumulation, symbiosis.

Introduction

Arbuscular mycorrhiza (AM), formed by roots of vascular plants and fungi of the order Glomerales, is the most widespread terrestrial symbiosis (Smith and Read, 1997[32]). An important feature of the AM symbiosis is the bidirectional exchange of nutrients between plant and fungal cells. AM fungi can deliver up to 80% of plant P, 25% N, 10% K, 25% Zn and 60% Cu (Marschner and Dell, 1994[18]) and, in turn, obtain between 10 and 25% of plant assimilates (Jakobsen and Rosendahl, 1990[16]). Smith and Smith (1990[33]) suggested that symbiotic nutrient transfer involves proton pumps located in plant and fungal membranes at the mycorrhizal interface, and cytochemical studies have shown that a plant H$^+$-ATPase is active in the periarbuscular membrane surrounding arbuscules in root cortical cells (Marx et al., 1982[19]; Gianinazzi-Pearson et al., 1991[11]). Regulation of proton pump activity at the transcriptional level in AM is suggested from three previous investigations. Murphy et al. (1997[22]) isolated a cDNA fragment from barley with similarity to H$^+$-ATPase-encoding genes and reported greater accumulation of the corresponding transcript in mycorrhiza as compared to control roots. Gianinazzi-Pearson et al. (2000[9]) detected mycorrhiza-induced promoter activity of two P-type H$^+$-ATPase genes, *Pma*2 and *Pma*4, in arbuscule-containing cells of tobacco roots. Recent analysis of tomato ATPase genes by Ferrol et al. (2002[6]) revealed for *Lha*2 up-regulation, but for *Lha*1 down-regulation during colonization with an AM fungus. In all cases, however, the genes were also expressed in other root tissues and in non-mycorrhizal roots.

In the present work, mycorrhiza-regulated H$^+$-ATPase genes were investigated in *Medicago truncatula*, a model plant for molecular analyses of plant-microbe interactions (www.medicago.org.). Three members of an H$^+$-ATPase gene family were identified of which one, *Mtha*1, turned out to be induced in AM roots. Gene expression profiling and *in situ* localization suggested that *Mtha*1 induction is specific to arbuscule-containing root cells and that it does not occur in other plant tissues.

Materials and Methods

Plant material

Medicago truncatula cv. Jemalong line A17 seeds were treated with concentrated sulphuric acid for 10 min, rinsed several times with water and sterilized with 12% sodium hypochlorite for 5 min. The seeds were then washed thoroughly with sterile water and spread on wet Whatman paper GB002 (Schleicher and Schuell, Dassel, Germany) in Petri dishes. Seeds were germinated at 4 °C in the dark and after three days, seedlings were planted into 100 ml substrate (1 : 1 expanded clay/vermiculite) and grown under constant conditions for one week (220 µE m^{-2} s^{-1} for 16 h; 22 °C; 65% humidity). For cloning and expression analyses of H$^+$-ATPase-encoding genes, inoculation with AM fungi in two independent experiments was performed as described by Dumas-Gaudot et al. (1994[4]), with two commercially available inocula (Biorize SARL, Dijon, France) containing

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either *Glomus mosseae* (Nicol and Gerd) Gerd. and Trappe (BEG 12) alone or a mixture of different AM fungi. Plants were fertilized weekly with 2 ml Long Ashton solution containing 0.13 mM NaH_2PO_4 per 100 ml substrate (Hewitt, 1966[15]). Roots were harvested, samples stained with trypan blue and mycorrhiza parameters evaluated following the method of Trouvelot et al. (1986[36]) (www.dijon.inra.fr/bbceipm/Mychintec/NewFiles/New.html).

Southern blot analysis

Genomic DNA was extracted from *M. truncatula* leaf material following the method of Dellaporta et al. (1983[3]). 10 µg DNA was cut with *Eco*RI, separated by agarose gel electrophoresis (0.8% agarose), capillary transferred onto a nylon membrane (Hybond N+; Amersham Bioscience, Freiburg, Germany) and hybridized under standard conditions at 60 °C (Sambrook et al., 1989[30]). The probe for hybridization was labelled by PCR in 20 µl using 1 µM M13 universal and reversal primers, 1 U of *Taq* polymerase (Gibco-BRL, Karlsruhe, Germany) and 0.1 mM dig-11-dUTP according to the protocol of Roche Diagnostics (Mannheim, Germany).

PCR techniques

RNA was extracted after 26 days growth from inoculated and non-inoculated root samples or from shoots with developing flowers and seeds (Franken and Gnädinger, 1994[7]). 5 µg total RNA were treated with DNAse (Bauer et al., 1993[1]) and used for cDNA synthesis with an oligo$(dT)_{15}$ as primer in 20 µl volume following the protocol of the supplier of the MMLV-reverse transcriptase (Promega, Mannheim, Germany). The cDNA was diluted 1 : 10 in water and 1 µl was directly used for PCR in 20 µl volume with 1 U *Taq* polymerase (Gibco-BRL), 200 µM dNTPs and 1 µM of respective primers. Sequences of primer pairs and annealing temperatures are summarized in Table **1**. Amplifications were carried out on the Trio-Thermoblock (Biometra, Göttingen, Germany) for 5 min at 95 °C followed by 30 cycles (30 s at 94 °C, 30 s at the specific annealing temperature and 30 s at 72 °C) and a final extension for 5 min at 72 °C. Amplification products were directly analysed by 2% agarose gel electrophoresis and, in addition, cloned and 5 clones each sequenced for confirming the specificity of the primer pairs for the three *Mtha* genes.

The primer pair HAol1 and HAol2 (Harper et al., 1994[13]) was used to clone ATPase-encoding genes. Fragments were cut from 2% agarose gels and purified using the Geneclean system (BIO101, Dianova, Hamburg, Germany). 5′- and 3′-RACE techniques were carried out according to the instructions of Gibco/BRL with 5 µg total RNA as starting material using the primers 5RACE1 or an oligo(dT) for cDNA synthesis and 5RACE2 or 3RACE1 for subsequent PCR. All PCR fragments were cloned into the pGEM-Teasy vector (Promega) according to the instructions of the supplier. After transformation of *E. coli* XL1-Blue competent cells, recombinant clones were tested by PCR with the corresponding primer pairs.

In order to follow RNA accumulation of the three *Mtha* genes, RT-PCR was carried out using the corresponding primer pairs (Table **1**) and the same conditions as described above. Competitive PCR with an exogenous internal standard was carried out using the PCR MIMIC™ construction system (Clontech, Heidelberg, Germany). The competitor fragment was synthesized using the composite oligonucleotides MIMIC.for and MIMIC.rev. For competitive amplifications, 0.1 attomol of the internal standard was added to the cDNA, and PCR was carried out with the *Mtha*1-specific primers Mtha1for.ol and Mtha1rev.ol under conditions described above. PCR amplifications were carried out in parallel using the primer pair Mtgapfor and Mtgaprev, specific for the *M. truncatula* gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 1 Primer sequences and annealing temperatures

HAol1	tbt gyw sbg aya aga cbg gba cby tba c	50 °C
HAol2	gcn tcd tty acy ccn tcy ccy gtc at	50 °C
Mtha1for.ol	caa gga ttt ggt tgt act aat g	68 °C
Mtha1rev.ol	cag gta ctt cct gtc tag ca	68 °C
Mtha2for.ol	tgc tga tgc cgt tgt ttt gat g	68 °C
Mtha2rev.ol	ctt ctt ggt atg cta cag	68 °C
Mtha3for.ol	gaa gga tta tgt cat cct tct t	65 °C
Mtha3rev.ol	cag gta ttt cct gtc tag ca	65 °C
MIMIC.for	caa gga ttt ggt tgt act aat gcg caa gtg aaa tct cct cc g	68 °C
MIMIC.rev	cag gta ctt cct gtc tag cat tga gtc cat ggg gag ctt t	68 °C
Mtgapfor	caa gca aca gaa gca ata tga aca	58 °C
Mtgaprev	ctt tga tgc caa ggc tgg aat tgc	58 °C
5RACE1	gta agc ttg tta aga gtt aat gtg	42 °C
5RACE2	caa ata cga atc gaa cag atg cag	60 °C
3RACE1	gca gat tct caa tct tgc ac	60 °C

Northern blot analysis

Five µg total root RNA were run on 1.4% formaldehyde agarose gels and transferred to Hybond N+ membranes (Amersham Bioscience) by capillary blotting (Sambrook et al., 1989[30]). Membranes were stained with methylene blue (30% [w/v] in 0.3 M NaAc, pH 5.2) and washed with sterile water. After monitoring the amounts of RNA, the dye was removed with 1% SDS, 1 × SSC, and membranes were hybridized at 65 °C under standard conditions (Sambrook et al., 1989[30]) to the *Mtha*1 cDNA fragment PCR labelled as described for Southern blot analysis.

In situ *hybridization*

Plants inoculated with *Glomus intraradices* Schenck and Smith were grown as described above. After seven weeks, mycorrhizal root pieces were fixed with 3% (w/v) paraformaldehyde in PBS (135 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4). After dehydration through a graded ethanol series, material was embedded in Paraplast (Sigma, Deisenhofen, Germany). Longitudinal sections (8 µm thick) were mounted on poly-L-lysine-coated slides, deparafinised and rehydrated. After rinsing in 10 mM Tris-HCl (pH 8.0), sections were incubated for 15 min with 1 µg/ml Proteinase K (in TE) at 37 °C and then for 1 h with 1% bovine serum albumin (BSA) in 10 mM Tris-HCl (pH 8.0). After acetylation, sections were dehydrated through a graded ethanol series and air-dried. For hybridization, sense or antisense RNA of the cDNA fragment *Mtha*1 was DIG-labelled according to the protocol of Roche Diagnostics. Root sections were incubated in a humid box at 45 °C overnight with a solu-

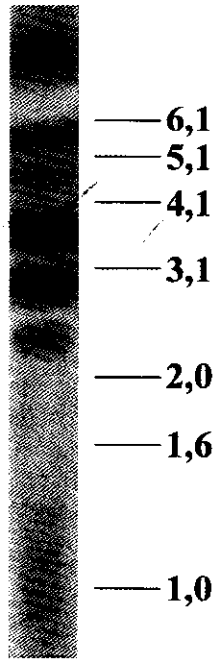

Fig. 1 Southern blot analysis. Genomic DNA of *M. truncatula* was digested with *EcoR*I, transferred to a membrane and hybridized to the *N. plumbaginifolia* gene *Npha2* (*Pma2*) as probe. Fragment lengths of a size marker are indicated.

tion of 0.3 M NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1 × Denhardt's solution, 50% formamide, 2 mg/ml tRNA, and 200 U/ml RNAse inhibitor containing denaturated sense or antisense probe. After incubation for 30 min with 20 µg/ml RNAse A at 37 °C, sections were washed three times for 30 min with 0.2 × SSC at 50 °C. Immunological detection of DIG-labelled RNA hybrids was performed with anti-DIG-fab fragment conjugated with alkaline phosphatase (Roche Diagnostics) according to the supplier's protocol, with nitro-tetrazolium blue chloride and 5-bromo-4-chloro-3-indolyl phosphate in the presence of 1 mM levamisol (all chemicals by Sigma). Hybridized sections were analysed by bright field microscopy using a Zeiss "Axioskop" microscope (Zeiss, Jena, Germany). Pictures were taken with a CCD camera (Sony, Tokyo, Japan) and processed through the Photoshop 4.0 program (Adobe, Seattle, MA).

Sequencing and computer analysis

Plasmid DNA was isolated by the miniprep method (Sambrook et al., 1989[30]) and sequenced on the sequencing automate ALF Express (Amersham Bioscience) using Cy5™-labelled M13 universal and reverse primers 18.1 and 18.2 (Appligene/Oncor, Heidelberg, Germany). Sequence analyses were carried out with the DNA Star program package using data from the EMBL/GeneBank. Nucleotide sequence data are deposited in the EMBL database under the AC numbers AJ132892 (*Mtha*1 cDNA clone), AJ132893 (*Mtha*2 cDNA fragment) and AJ132894 (*Mtha*3 cDNA fragment). The alignment of protein data was carried out by ClustalW (Thompson et al., 1994[35]) using sequences from the SWISSPROT database, and phylogenetic analysis with the program PUZZLE (Version 4.0.2; Strimmer and von Haeseler, 1996[34]). Based on the results, a distance tree was constructed using the program TREEVIEW (Version 1.5.3; Page, 1996[24]).

Results

Identification of Medicago truncatula *ATPase genes*

The *Npha*2 gene (*Pma*2) of *Nicotiana plumbaginifolia* (Perez et al., 1992[27]) was used as a probe to investigate whether H^+-ATPase genes are organized in a gene family in *M. truncatula*. Six clear signals with different intensities appeared in the Southern blot analysis of *M. truncatula* genomic DNA under low-stringency conditions (Fig. **1**). RT-PCR with degenerate primers for plant H^+-ATPases (Harper et al., 1994[13]) was carried out with cDNA from *Glomus mosseae*-inoculated roots in order to clone cDNA fragments of H^+-ATPase genes transcribed in arbuscular mycorrhizal roots of *M. truncatula*. Amplification resulted in 800 bp PCR products that were cloned and sequenced. After analysing 10 different clones, three different sequences were obtained which showed more than 70% identity on nucleic acid level to plant genes encoding other plasma membrane H^+-ATPases, indicating that at least three genes were expressed in *M. truncatula* AM (*Mtha*1 – 3 according to the nomenclature used for *Arabidopsis thaliana* in Harper et al., 1994[13]). *Mtha*1 and *Mtha*2 cDNA fragments shared 71.2% identical nucleotides, while similarity of the *Mtha*3 cDNA fragment to *Mtha*1 and *Mtha*2 was about 65%.

RNA accumulation of Mtha*1 – 3*

Specific primer combinations for each H^+-ATPase cDNA fragment were deduced to screen for changes in transcription levels of the corresponding genes in control and AM roots in two independent experiments, where *G. mosseae* or a mixed inoculum have been used (mycorrhization intensity = 45% with both inocula). The specificity of each primer pair was confirmed using the plasmids as templates (data not shown). Amplification of *Mtha*1 fragments only gave the expected 450 bp RT-PCR products with cDNA from roots colonized by AM fungi but not with non-colonized root cDNA (Fig. **2B**). Specific amplification of cDNA fragments belonging to the two other H^+-ATPase genes and with the degenerated primer pair resulted in the expected 320 bp bands of equal intensities from all templates (Figs. **2A, C, D**). This indicated that the genes *Mtha*2 and *Mtha*3 are constitutively expressed in roots, while RNA accumulation of *Mtha*1 is regulated by AM development. Northern blot experiments were carried out as further proof for specific *Mtha*1 RNA accumulation. Total RNA of control and *G. mosseae*-inoculated roots were hybridized to the 800 bp RT-PCR fragment belonging to *Mtha*1. A signal at 3.2 kb appeared only in the mycorrhiza RNA lane, whereas no signal could be detected in the control (Fig. **3**).

In a third experiment (*G. mosseae* mycorrhization intensity = 42%), competitive RT-PCR with an exogenous standard was used to investigate whether the change of *Mtha*1 transcript levels resulted directly from mycorrhiza development or from the indirect mycorrhizal effect of improved phosphate availability to the plant. Template RNA was extracted from roots of non-inoculated *M. truncatula* fertilized with Long Ashton solution containing either 1.3 mM or 0.13 mM NaH_2PO_4, or of AM plants fertilized with the lower P_i concentration. Competitive amplification for the *Mtha*1 gene was only successful using