Curr Genet (2000) 37: 112–118

ORIGINAL PAPER

Nuria Ferrol · José Miguel Barea
Concepción Azcón-Aguilar

The plasma membrane H^+-ATPase gene family in the arbuscular mycorrhizal fungus *Glomus mosseae*

Received: 13 August / 24 October 1999

Abstract To identify genes that encode plasma membrane H^+-ATPases in the arbuscular mycorrhizal fungus *Glomus mosseae* two sets of degenerate primers matching highly conserved motifs present in all plant and fungal ATPases were designed. Nested PCR-amplification of *G. mosseae* genomic DNA using the designed degenerate primers was carried out. Sequence analysis of the cloned PCR products identified five different clones (GmHA1, GmHA2, GmHA3, GmHA4 and GmHA5) encoding putative plasma membrane H^+-ATPases. Comparison of the deduced amino-acid sequences of GmHA1–GmHA5 indicate that GmHA1, GmHA3 and GmHA4 are highly identical, while GmHA2 and GmHA5 are more divergent. The evolutionary and functional significance of the divergence found among the different members of the H^+-ATPase gene family in *G. mosseae* is discussed.

Key words Arbuscular mycorrhizas · Evolution · *Glomus mosseae* · Plasma membrane H^+-ATPase genes

Introduction

The plasma membrane H^+-ATPase is an electrogenic proton pump which plays important energetic and regulatory roles in the cells of plants and fungi. The activity of this enzyme generates an electrochemical gradient of H^+ across the cell plasma membrane that drives a number of secondary transport systems, including those responsible for the translocation of cations, anions, amino acids and sugars (Tanner and Caspari 1996). The plasma membrane H^+-ATPase also contributes to the maintenance of the extracellular and intracellular pH (Smith and Raven 1979), and it has been proposed that regulation of the plasma membrane H^+-ATPase may mediate a broad range of physiological responses which play a central role in growth and development (Serrano 1984).

Plasma membrane H^+-ATPases belong to the evolutionary related family of P-type cation-translocating ATPases, which are characterized by the formation of a phosphorylated intermediate during the catalytic cycle, inhibition by vanadate, and structural similarity in several conserved domains (Serrano 1989). Other members included in this family are the plasma membrane Na^+-ATPase of fungi, the Ca^{2+}-ATPases of plants, animals and fungi, and the H^+/K^+- and Na^+/K^+-ATPases of animals (Palmgren and Axelsen 1998). Molecular studies of plasma membrane H^+-ATPases have revealed that in plants such as *Arabidopsis* (Harper et al. 1990), tomato (Ewing and Bennett 1994) and tobacco (Perez et al. 1992; Moriau et al. 1993), and in the yeasts *Saccharomyces cerevisiae* (Serrano et al. 1986; Schelesser et al. 1988) and *Schizosaccharomyces pombe* (Ghislain et al. 1987; Ghislain and Goffeau 1991), these enzymes are coded by several genes. While numerous biochemical and molecular studies on H^+-ATPases have been carried out in higher plants and yeasts, the information about the structure and function of plasma membrane H^+-ATPases in other fungi is scarce. There is a report of the molecular characterization of the plasma membrane H^+-ATPase of the parasitic rust fungus *Uromyces fabae* (Struck et al. 1998), but nothing is known about these genes in the mutualistic fungi forming arbuscular mycorrhizas.

Arbuscular mycorrhizal (AM) fungi, belonging to the Zygomycota, are obligate biotrophs that form mutualistic symbioses with most plant species. The benefits of mycorrhizal symbiosis to plant development depends mainly on the ability of the fungal mycelium to take up

Communicated by K. Esser

N. Ferrol (✉) · J.M. Barea · C. Azcón-Aguilar
Departamento de Microbiología del Suelo y Sistemas Simbióticos,
Estación Experimental del Zaidín,
Consejo Superior de Investigaciones Científicas,
Profesor Albareda 1, 18008 Granada, Spain
e-mail: nferrol@eez.csic.es,
Tel.: +34-958-121011
Fax: +34-958-129600

nutrients from the soil and to transfer them to the symbiotic roots in exchange for carbon compounds (Azcón-Aguilar and Bago 1994). Because the transport of many solutes into and out of the cells requires the action of the plasma membrane H^+-ATPase, it is likely that in the AM symbiosis the fungal plasma membrane H^+-ATPase plays an important role in the absorption of nutrients by the AM fungus. A positive correlation has been observed between the ATPase activity and ^{32}P uptake in hyphae from germinated spores of the AM fungus *Gigaspora margarita* (Lei et al. 1991). Moreover, cloning of a high-affinity phosphate transporter of the AM fungus *Glomus versiforme* that is operated by H^+ symport (Harrison and van Buuren 1995) supports this hypothesis. Cytochemical studies of mycorrhizal roots have also shown the presence of ATPase activity in the plasma membrane of the intercellular fungal hyphae (Gianinazzi-Pearson et al. 1991). In spite of the importance of the fungal plasma membrane H^+-ATPase in the functioning of the AM symbiosis, nothing is known about the genes coding for this enzyme. This is due to the fact that molecular studies of AM fungi are difficult because of their obligate, biotrophic nature. In the present work by using a PCR-based approach we have identified five partial genomic clones encoding P-type ATPases that potentially represent five isoforms of the plasma membrane H^+-ATPase in the AM fungus *Glomus mosseae*.

Materials and methods

Biological material

Sporocarps of *G. mosseae* (Nicol. & Gerd.) Gerd. & Trappe (BEG 119) were obtained from pot cultures of *Medicago sativa* L. grown in a soil/sand/vermiculite (1/1/1, v/v/v) mixture. They were collected by soil wet-sieving (Gerdemann and Trappe 1963) and sporocarps were sorted out with a fine forceps under a dissecting microscope, transferred into a Petri dish containing wet filter paper and broken with a needle. Spores released from the sporocarps were hand-picked with ultra-fine forceps under a dissecting microscope, placed in a sterile Eppendorf tube containing 200 µl of sterile ultra-pure water and rinsed four times with fresh sterile ultra-pure water.

The extra-radical mycelium of *G. mosseae* was collected from mycorrhizal plants by scraping the roots placed in sterile water and passing this water through a 50 µm-mesh sieve. The mycelium was hand-picked with a forceps under a dissecting microscope, immediately frozen in liquid nitrogen and stored at −80 °C.

DNA extraction

To extract genomic DNA from *G. mosseae* spores, about 1000 spores were crushed with a sterile disposable pestle in a lysis buffer containing 50 mM Tris pH 8.0, 25 mM EDTA, 0.5% N-lauryl sarcosinate, 2% SDS, 1 mg/ml of proteinase K and 2% 2-mercaptoethanol. After incubation for 45 min at 65 °C, 0.25 vol of 5 M NaCl were added and the mixture was incubated for 30 min on ice. Nucleic acids were extracted and precipitated following standard procedures (Sambrook et al. 1989).

Genomic DNA of the extra-radical mycelium of *G. mosseae* was extracted according to the method of Murray and Thompson (1980).

PCR-amplification and DNA sequencing

Two 20-mer degenerate oligonucleotides, designated as P1 (sense): TG(C/T)(T/A)(C/G)(T/C)GA(T/C)AA(A/G)AC(T/C)GGIAC and P2 (antisense): TT(G/A/C)AC(A/C/T)CC(G/A)TC(A/C/T)CCIGTCAT (I = Inosine) were synthesized (Boehringer, Mannheim, Germany) and used for PCR-amplification of genomic DNA. These primers were designed to match two highly conserved motifs present in all sequenced fungal H^+-ATPases (Serrano 1989). Amplifications were carried out in a final volume of 50 µl containing 1 × PCR buffer (10 mM TrisHCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/ml of gelatin), 10 ng of genomic DNA, 0.2 mM of each dNTP, 100 pmol of each primer and 1 unit of *Taq* DNA polymerase (Appligene, Oncor, USA). PCR was performed in an automated thermal cycler (Gene Amp PCR system 2400, Perkin Elmer, Foster City, Calif.) with an initial denaturation for 5 min at 94 °C, followed by 35 cycles with denaturation for 1 min at 94 °C, annealing for 2 min at 55 °C and extension for 2.5 min at 72 °C, followed by a 7-min final extension at 72 °C. The P1-P2 PCR product was subjected to a nested PCR. For this puspose, two degenerate 20-mer primers, P3 (sense): GT(C/T)AA(A/G)GG(A/T)GC(T/C)CC(A/T)(C/T)T(T/G/A)TT and P4 (antisense): GC(G/A)TC(A/G)CC(A/G)CTIA(G/A/T)CAT(T/C)TT (I = Inosine), were designed. Nested PCR was carried out under the conditions described above, but using 0.5 µl of the initial PCR product and the following pairs of primers: P1/P4 and P3/P2.

The amplification products were separated electrophoretically on 1.6% agarose, stained with ethidium bromide and the expected bands were excised with a scalpel. DNA fragments were isolated from the gel with the Wizard PCR Prep DNA Purification System (Promega, Madison, USA) and cloned into the pGEM-T vector (Promega, Madison, USA), following the manufacturer's protocols. Nucleotide sequences were determined by using *Taq* polymerase cycle sequencing and an automated DNA sequencer (Perkin-Elmer ABI Prism 373). All clones were sequenced in both strands using universal and reverse primers.

Sequence analyses

The sequences were analyzed using the Genetics Computer Group (Madison, Wis.) package (version 9.0). The sequence data were compared to gene libraries (EMBL and GenBank) with BLAST (Altschul et al. 1990) and FASTA (Pearson and Lipman 1988) programs. Amino-acid sequence comparisons were made with the BESTFIT program. Multiple sequence alignments of translated gene sequences were carried out with the program CLUSTALW (version 1.5; Thompson et al. 1994). Genetic distances were estimated by using the Kimura two-parameter method employed by PHYLIP (Felstein 1993). Phylogenetic analyses were performed by the neighbor-joining and evolutive parsimony methods by using PHYLIP (Felstein 1993). The relative support for groups was determined based upon 100 bootstrap trees.

Southern hybridization

G. mosseae genomic DNA was digested with *Eco*RI, fractionated on a 0.8% agarose gel, and blotted onto nylon membranes as described by Southern (1975). The P1–P4 region of GmHA1 to GmHA5 were labelled with DIG-11-dUTP by PCR and used as probes. Hybridizations were performed overnight at 42 °C in 50% formamide, 5 × SSC, 2% blocking reagent (Boehringer, Mannheim, Germany), 0.1% N-laurylsarkosine, and 0.02% sodium dodecyl sulphate (SDS). Membranes were washed twice for 5 min at room temperature with 2 × SSC plus 0.1% SDS and twice for 15 min at 68 °C with 0.1 × SSC and 0.1% SDS. Blots were developed by following the manufacturer's instructions for the chemiluminescent detection of digoxigenin-labelled probes with alkaline phosphatase-antibody conjugates (Boehringer Mannheim).

Nucleotide sequence accession numbers

The sequences of the GmHA genes were deposited in the EMBL database under accession no. AJ133839 through AJ133843.

Results

Cloning and characterization of genes encoding plasma membrane H^+-ATPases in *G. mosseae*

As a first step to identify genes that encode plasma membrane H^+-ATPases in *G. mosseae*, two degenerate oligonucleotides (P1 and P2) were designed to match two highly conserved motifs (CSDKTG and MTGDGV) found at the catalytic site contained within the large central hydrophilic loop present in all plant and fungal H^+-ATPases (Serrano 1989). In all characterized fungal H^+-ATPases, these primers flank a 790-bp region that does not include any intron. This primer pair was used to prime PCR-amplification of genomic DNA isolated from spores of *G. mosseae*. Amplified DNA fragments approximately 800 bp in length were subcloned into a plasmid vector and ten of the resulting clones were partially sequenced. However, sequence analysis revealed that these clones all contained DNA fragments that did not show significant homology with any gene present in the sequence databases.

To increase the specificity of the PCR, a new set of degenerate oligonucleotides (P3 and P4) was designed to perform a nested PCR using the P1-P2 PCR product as a DNA template. These primers were based on the DNA sequence encoding two conserved motifs (KGAPLF and KMLTGD) within the P1/P2 domain in all fungal H^+-ATPases (Serrano 1989). When the P1-P4 and P3-P2 primer pairs were used to prime PCR-amplification of the P1-P2 PCR product, bands of approximately 550 and 500 bp, respectively, were obtained. These amplified DNA fragments were subcloned and 20 of the resulting clones of each reaction were sequenced. Sequence analysis showed that there were representatives of five different genes, designated as GmHA1, GmHA2, GmHA3, GmHA4 and GmHA5 (Fig. 1). Over the P1-P4 region, four identical subclones were found to correspond to GmHA1, three to GmHA2, four to GmHA3, four to GmHA4 and five to GmHA5. Over the P3/P2 region, the sequenced clones overlap the five different genes identified with the P1-P4 primer set. Four of them were found to correspond to GmHA1, three to GmHA2, six to GmHA3, four to GmHA4 and three to GmHA5. It appeared that a high level of fidelity was achieved in the amplifications, since all the subclones matching each gene from GmHA1 through to GmHA5 were at least 99% identical.

Comparisons of the deduced amino-acid sequences of GmHA1–GmHA5 over the region P1-P2 indicates that GmHA1, GmHA3 and GmHA4 show close similarity, while GmHA2 and GmHA5 are more divergent (Table 1). Additionally, the G + C content of GmHA5 (40%) was low in comparison to the G + C contents of the other GmHA genes (53, 54, 60 and 57% for GmHA1 to GmHA4, respectively). The deduced amino-acid sequences of the amplified region of GmHA1 through GmHA5 showed the highest identity with fungal and plant plasma membrane H^+-ATPases (Table 2).

```
GmHA1   CSDKTGTLTKNKLSLAEPYTVEGVDAEDLMLTACLAASRKKKGLDAIDKAF-------LK
GmHA2   CCDKTGTLTANKLSIRDPYVAEGQDVDWMMAVAALASSHNLKSLDPIDKVT-------IL
GmHA3   CSDKTGTLTKNKLSLAEPYTVAGVDPEDLMLTACLAASRKKKGMDAIDKAF-------LK
GmHA4   CSDKTGTLTKNKLSLSEPFCVEGVDPDDLMLTACLAASRKKKGIDAIDKAF-------LK
GmHA5   CCDKTGTLTLNELTFDEPYLCPGYTKDDILLFSYLSAEPGAN--DPIETAVRFAAETDLE
        *:******* *:*:: :*:   *   : ::  : *:::   :  *:*:::        :

GmHA1   SLRYYPRAKSVLSK-YKVLEFHPFDPVSKK-VTAVVESPQGERIICVKGAPLFVLRTVEE
GmHA2   TLKRYPGAREILQQGWKTESFTPFNPVSKR-ITSVCRLN-GDKYTCAKGAPSAILKLTNC
GmHA3   SLKFYPRAKGVLSK-YKVIDFHPFDPVSKK-VTAVVESPQGERIICVKGAPLFVLKTVEE
GmHA4   ALRHYPLAKNVLSK-YAVLDFQPFDPVSKK-VQVTVESPQGERIICVKGAPMAVLNTVAQ
GmHA5   ILQSRP-NKHEVPG-YKVTGFVPFNPNTKMSYATVIDNNTKEVFKVAKGAPQVIIKLVGG
         *:  *  :  :   : :  * **:* :*     :      :    :****  ::: :

GmHA1   DHPIPEEVATDYKNKVAEFATRGFRSLGVARRR-GE-GHWEILGIMPCSDPPRHDTAKTV
GmHA2   S----DETRQLCKEKAQEFARRGFRSLGVAVKK-ND-EDWVLLGLLSMFDPPREDTAQTI
GmHA3   DHPIPDEVDQAYKNKVAEFATRGFRSLGVARKR-GE-GSWEILGIMPCSDPPRHDTARTV
GmHA4   DHPIPEEIDQAYKNKVAEFATRGFRSLGVARKR-GQ-GSWEILGIMPCSDPPRHDTYRTI
GmHA5   N--------DDAVHAVNSLAARGLRALGIARTVPGDLETFDLVGMITLLDPPRPDSAETI
        :           : : ::* **:*:**:*    ::   : ::*:::  **** *: :*:

GmHA1   NEASTLGLSIKMLTGDAVGIARETSRQLGLGTNIYNAEKLGLGGGGEMPGSEVYDFVEAA
GmHA2   LEASHLGVPVKMLTGDAIAIAKETCKMLSLGTKVYNSERLIHGG---LAGTVQHDFVERA
GmHA3   NEAKSLGLSIKMLTGDAVGIARETSRQFGLGTNIYNAERLGLGGGGDMPGSEVYDFVEAA
GmHA4   NEAKNLGLSIKMLTSDAVGIARETSRQLGLGTNVYNSERLGLGGGGDMPGSEVYDFVEAA
GmHA5   RRCGEYGVEVKMITGDQLIIAKEVAHRLGMNRVILDAGYLVDPE---KSDEEVTKNCERA
         ::   *: :**:*:* : **:*::: ::::  : ::  *        ::    :  * *

GmHA1   DGFAEVFPQHKYNVLQILQQR-GYLVAMTGDGV
GmHA2   DGFAEVFPGHKYTVVEMLQQR-GHLTAMTGDGV
GmHA3   DGFAEVFPQHKYNVVEILQQR-GYLVAMTGDGV
GmHA4   DGFAEVFPQHKYSVVEILQQALATLFAMTGDGV
GmHA5   DGFAQVIPEHKYRVVELLQKR-GLLVGMTGDGV
        ****:*:* *** *:::**:  : * :******
```

Fig. 1 Alignment of the deduced amino-acid sequences of GmHA1, GmHA2, GmHA3, GmHA4 and GmHA5. *Dashes* represent gaps introduced into the alignment, "*" indicates identical amino-acids and ":" indicates similar amino-acids

Table 1 Percentage of identity between the deduced amino-acid sequences of GmHA1, GmHA2, GmHA3, GmHA4 and GmHA5. Identities were calculated using the Bestfit Program in the GCG software package

Amino acid	GmHA2	GmHA3	GmHA4	GmHA5
GmHA1	52	91	83	38
GmHA2		54	53	38
GmHA3			86	39
GmHA4				37

GmHA1, GmHA3 and GmHA4 had highest homology with the plasma membrane H^+-ATPase of *Neurospora crassa* (91, 94 and 85% identity, respectively) and *Ajellomyces capsulata* (91, 92 and 85%, respectively); high identity levels were also found with plasma membrane H^+-ATPases from yeasts (74–85%), but lower levels were found with algae (< 47%) and plant (< 44%) H^+-ATPases. GmHA2 showed the highest sequence homology to the plasma membrane H^+-ATPase of the filamentous fungus *Emericella nidulans* (76% identity) and lower to yeast (< 55%) and plant (< 46%) H^+-ATPases. The highest homology of GmHA5 was obtained with the plasma membrane H^+-ATPases of algae and plants (47 and 45% identity, respectively), sharing only about 40% identity with the fungal genes. Identity of GmHA1 through GmHA5 to other P-ATPases was lower than 24%. These data suggest that the five cloned partial genes encode five putative plasma membrane H^+-ATPase isozymes in *G. mosseae*.

To verify if each PCR clone represented a gene from *G. mosseae*, blots of *G. mosseae* genomic DNA were probed with the P1-P4 region of each clone at high stringency conditions. Although the PCR was carried out with genomic DNA isolated from spores, Southern-blot analyses were performed using genomic DNA isolated from external hyphae because of the difficulty in collecting enough DNA from spores to do these analyses. As external hyphae could be contaminated with plant tissue, genomic plant DNA was also tested for hybridization with the GmHA probes. Plant DNA did not cross-hybridize with any of the fungal genes (data not shown). Figure 2 shows that under these conditions GmHA1, GmHA2, GmHA3 and GmHA5 hybridized to a single genomic fragment and that GmHA4 hybridized to two bands of almost the same intensity, which suggests that there may exist an additional related gene in the *G. mosseae* genome.

Molecular evolution of GmHA genes

To examine the evolutionary relationships among members of the ATPase gene family, phylogenetic trees were generated from multiple aligned sequences by using evolutionary parsimony and neighbor-joining methods. As the results obtained from both methods are highly congruent, only results obtained from the neighbor-joining method are shown. The phylogenetic tree was constructed by using the H^+-ATPase gene of the archea *Methanococcus jannaschii* as an outgroup. The molecular cladogram shows that the GmHA genes are members of a large gene family that includes the H^+-ATPases of fungi, plants and algae, and that the GmHA genes fall into three different gene subgroups (Fig. 3). This tree also shows that the primary divergence among H^+-ATPases appears to divide the fungal genes, with the exception of GmHA5 and the gene of a biotrophic

Table 2 Percentage of identity between the deduced amino-acid sequences of GmHA1, GmHA2, GmHA3, GmHA4 and GmHA5 and plasma membrane H^+-ATPases from different organisms. Identities have been calculated using the Bestfit Program in the GCG software package

Organism	Gene	Acc. no.	GmHA1	GmHA2	GmHA3	GmHA4	GmHA5
Neurospora crassa	PMA1	P07038	91	54	94	85	39
Ajellomyces capsulata	PMA1	Q07421	91	55	92	85	40
Zygosaccharomyces rouxii	PMA1	P24545	85	53	85	77	40
Kluyveromyces lactis	PMA1	P49380	85	55	85	77	39
Saccharomyces cerevisiae	PMA1	P05030	84	55	84	77	40
Saccharomyces cerevisiae	PMA2	J04421	84	53	84	78	40
Candida albicans	PMA1	P28877	82	51	82	75	38
Pneumocystis carinii	PCA1	U65004	81	52	80	77	39
Schizosaccharomyces pombe	PMA1	P09627	77	51	78	74	42
Schizosaccharomyces pombe	PMA2	P28876	77	53	78	77	42
Emericella nidulans	PMA1	AF036763	54	76	52	54	35
Uromyces fabae	PMA1	AJ003067	35	44	35	37	40
Cyanidium caldarium	–	D88424	45	45	47	43	47
Dunaliella acidofila	DHA1	U54690	41	44	42	43	44
Dunaliella bioculata	PMA1	X73901	38	41	38	40	41
Nicotiana plumbaginifolia	PMA1	Q08435	40	42	38	41	44
Nicotiana plumbaginifolia	PMA3	Q08436	40	42	39	41	44
Nicotiana plumbaginifolia	PMA4	Q03194	42	46	41	40	45
Lycopersicon esculentum	LHA1	P22180	40	44	38	41	45
Lycopersicon esculentum	LHA2	P23980	41	45	38	42	45
Arabidopsis thaliana	AHA1	P20649	41	45	38	42	45
Arabidopsis thaliana	AHA2	P19456	40	45	41	44	45

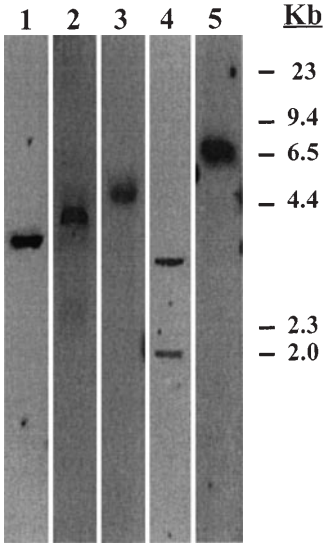

Fig. 2 Genomic Southern blots of *G. mosseae* genomic DNA hybridized at high strigency with the P1-P4 region of GmHA1 (*lane 1*), GmHA2 (*lane 2*), GmHA3 (*lane 3*), GmHA4 (*lane 4*) and GmHA5 (*lane 5*)

fungus (*U. fabae*), from the H^+-ATPase genes of plants and algae. The GmHA1, GmHA2, GmHA3 and GmHA4 sequences are grouped with a very high level of confidence (100% of bootstrap trees) in the fungal clade clustering the H^+-ATPase genes of yeasts and filamentous ascomycetes, with the GmHA genes placed closer to the genes of the filamentous ascomycetes. Separation of the clade containing GmHA2 and the gene of *E. nidulans* from the clade containing the other fungal genes was also strongly supported. The sequence GmHA5 is clustered in the clade grouping the H^+-ATPase genes of the biotrophic fungus *U. fabae*, algae and plants, although this topology was only supported by 65% of the bootstrap trees.

Discussion

Genetic characterization of AM fungi has been hampered by the inability to grow them in pure culture. Recently, molecular approaches based on the use of PCR have allowed the identification of ribosomal sequences in several AM species (Simon et al. 1993; Harney et al. 1997; Redecker et al. 1997), a nitrate reductase gene in two *Glomus* species (Kaldfor et al. 1994, 1998) and genes encoding glycerol-3-phosphate dehydrogenase, β-tubulin and two types of ATPases involved in transmembrane Ca^{2+} and Li^+/Na^+ transport in *Gigaspora rosea* (Franken et al. 1997). In the present report, by using a PCR-cloning approach based on the use of highly degenerate primers, we have isolated five partial genomic clones encoding P-type ATPases that potentially represent five isoforms of the plasma membrane H^+-ATPase in the AM fungus *G. mosseae*.

Analysis of the nucleotide and deduced amino-acid sequences of the five isoforms indicates that these genes display the highest homology to plasma membrane H^+-ATPases from other organisms. However, identities to genes encoding P-type ATPases involved in transmembrane Ca^{2+} and Li^+/Na^+ transport in the AM fungus *G. rosea* (Franken et al. 1997) was very weak (lower than 10%). This is not surprising because evolutionary studies of the P-type ATPase superfamily revealed that the various P-ATPases group together in a phylogenetic tree

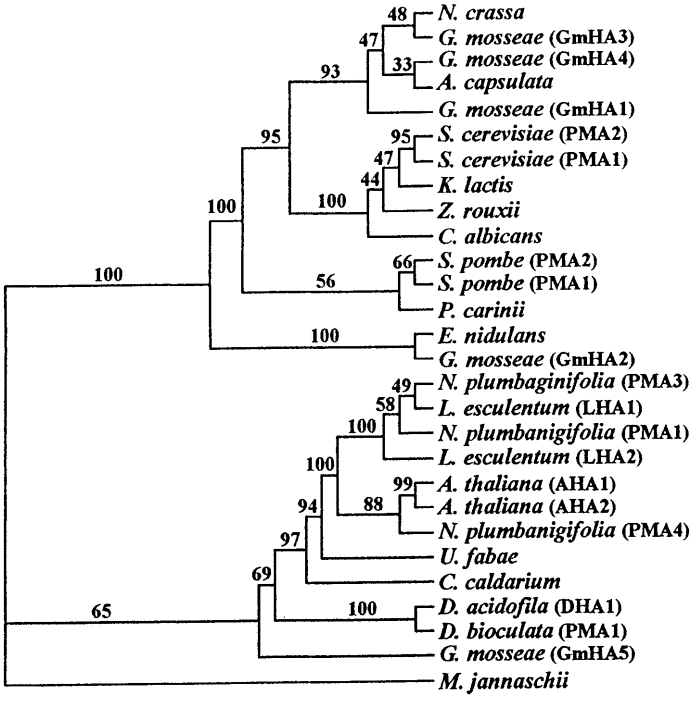

Fig. 3 Phylogenetic tree of the plasma-membrane H^+-ATPase genes represented in Table 2. The tree was constructed by using the neighbor-joining method, and the archea *Methanococcus jannischii* as an outgroup. *Numbers* represent the relative support for subgroupings based upon 100 bootstrap trees

according to substrate specificity, irrespective of the evolutionary distance between the parental species (Axelsen and Palmgren 1998).

The genes obtained in this study are divergent and fall into three different subgroups. The divergence found between the GmHA genes is much greater than the divergence observed between the different members of the ATPase gene family in other organisms. Although the gene tree was carried out with partial sequences, the evolutionary relationships found in this study are in agreement with those previously obtained using the full-length proteins (Sussman 1994; Rao and Slayman 1996). The phylogenetic analysis shows that the GmHA gene family has emerged from different duplication events. The close homology between GmHA1, GmHA3 and GmHA4 suggests that they could have arisen from two relatively recent gene-duplication events. The gene tree also indicates that GmHA5 and the ATPase gene of the biotrophic fungus *U. fabae* are more closely related to H^+-ATPase sequences from plants and algae than from fungi. This contrasts with what would be predicted from taxonomic criteria. Although the evolutionary significance of the divergency of GmHA5 has to be elucidated, it could be the result of the close association and long period of co-evolution of AM fungi and plants.

Molecular analysis of small-subunit rRNA sequences of AM fungi has dated their origin at 353–462 million years ago (Simon et al. 1993) while fossil records indicate that mycorrhizal symbioses were established more than 400 million years ago (Remy et al. 1994; Taylor et al. 1995). These findings are consistent with the hypothesis that arbuscular mycorrhizas facilitated the colonization of land by ancient plants by establishing a mutualistic symbiosis (Pirozynski and Dalpé 1989). Since then the AM fungi have probably coevolved with plants and the results of this long period of coevolution is reflected in the complex interactions taking place between both in extant AM symbiosis. Coevolutionary changes in the gene pools of both symbionts might account for overall accomodation and coadaptation of AM fungi and plants during the course of evolution. Coevolution between symbionts can result from either vertical transmission of novel inheritable traits acquired as a consequence of coadaptation or from horizontal gene transfer between partners (Pirozynski and Hawksworth 1988). Contradictory phylogenetic relationships or an anomalous G+C content in the gene of interest can provide evidence suggestive of horizontal gene transfer between distantly related species (Nielsen et al. 1998). Since the topology of the molecular cladogram suggests that GmHA5 has a different evolutionary origin from the other GmHA genes, but the same as the plant H^+-ATPase genes, we hypothesize that GmHA5 could have been horizontally transferred from the host plant to the AM fungus during the course of evolution. This hypothesis is supported by the observation that the G+C content of GmHA5 (40%) is definitively lower than in the other GmHA genes (53–60%) but is within the range of the plant H^+-ATPase genes (40–44%). Although a transfer of genetic material from plants to organisms other than plants has been claimed to be absent (Liberman et al. 1996), Hoffman et al. (1994) have recently reported the transfer of genetic material from plants to plant-associated fungi.

The divergence found between the GmHA genes highlights the variety of specialized physiological functions for which ATPases have evolved in AM fungi. As stated for the plant ATPase genes (Sussman 1994), the small sequence differences in amino-acids among the different isozymes may be important for generating proton pumps with unique catalytic properties tailored to the specific transport functions of the different fungal structures. Alternatively, the divergence among the different GmHA genes may indicate key differences in their regulatory properties. For *Arabidopsis*, whose plasma membrane H^+-ATPase is encoded in a multigene family composed of three subfamilies, it has been found that the isozymes that have been studied are expressed in a very specific way, which depends on the cell type, developmental stage and environmental conditions (Houlné and Boutry 1994; Sussman 1994; De Witt and Sussman 1995). Similarly, it could be hypothesized that members of the plasma membrane H^+-ATPase gene family of *G. mosseae* can be differentially expressed in the different fungal structures (spore germ tubes, appressoria, extra- or intra-radical hyphae, or arbuscules, etc.), depending on the physiological stage of the AM symbiosis. Based on the divergence found between GmHA5 and the genes of the free-living fungi, and the relatively closer similarity of GmHA5 to the genes of a biothrophic fungus and plants, it is tempting to speculate that this gene could be expressed in the symbiotic fungal structures. However, expression studies of the different GmHA genes are necessary to understand the physiological role of the different H^+-ATPase isozymes of *G. mosseae*.

Acknowledgements We are extremely grateful to Dr. Peter Jeffries for helpful comments on the manuscript. We also thank Drs. José Olivares and Berta Bago for helpful discussions and Mrs. Custodia Cano for technical assistance. This research was supported by the E.U., TMR Network ERB FMR XCT 960039.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Axelsen KB, Palmgren MG (1998) Evolution of substrate specificities in the P-type superfamily. J Mol Evol 46:84–101

Azcón-Aguilar C, Bago B (1994) Physiological characteristics of the host plant promoting an undisturbed functioning of the mycorrhizal symbiosis. In: Gianinazzi S, Schuepp H (eds) Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. Birkhauser Verlag, Basel, pp 47–60

De Witt ND, Sussman MR (1995) Immunocytological localization of an epitope-tagged plasma membrane proton pump (H^+-ATPase) in phloem companion cells. Plant Cell 7:2053–2067

Ewing NN, Bennett AB (1994) Assessment of the number and expression of P-type H^+-ATPase genes in tomato. Plant Physiol 106:547–557

Felstein J (1993) PHYLIP, Version 3.5, Department of Genetics, University of Washington, USA

Franken P, Lapopin L, Meyer-Gauen G, Gianinazzi-Pearson V (1997) RNA accumulation and genes expressed in spores of the arbuscular mycorrhizal fungus *Gigaspora rosea*. Mycologia 89:293–297

Gerdemann JW, Trappe JM (1963) Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. Trans British Mycol Soc 46:235–244

Ghislain M, Goffeau A (1991) The *pma1* and *pma2* H^+-ATPases from *Schizosaccharomyces pombe* are functionally interchangeable. J Biol Chem 266:18276–18279

Ghislain M, Schelesser A, Goffeau A (1987) Mutation of a conserved glycine residue modifies the vanadate sensitivity of the plasma membrane H^+-ATPase from *Schizosaccharomyces pombe*. J Biol Chem 262:17549–17555

Gianinazzi-Pearson V, Smith SE, Gianinazzi S, Smith FA (1991) Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhizas. V. Is H^+-ATPase a component of ATP-hydrolysing enzyme in plant-fungus interfaces? New Phytol 117:61–74

Harney SK, Edwards FS, Allen MF (1997) Identification of arbuscular mycorrhizal fungi from *Artemisa californica* using the polymerase chain reaction. Mycologia 89:547–559

Harper JF, Manney L, De Witt ND, Yoo MH, Sussman MR (1990) The *Arabidopsis thaliana* plasma membrane H^+-ATPase multigene family. J Biol Chem 265:13601–13608

Harrison MJ, van Buuren ML (1995) A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. Nature 378:625–629

Hoffman T, Golz C, Schieder O (1994) Foreign DNA sequences are received by a wild-type strain of *Aspergillus niger* after co-culture with transgenic higher plants. Curr Genet 27:70–76

Houlné G, Boutry M (1994) Identification of an *Arabidopsis thaliana* gene encoding a plasma membrane H^+-ATPase whose expression is restricted to anther tissues. Plant J 5:311–317

Kaldorf M, Zimmer W, Bothe H (1994) Genetic evidence for the occurrence of assimilatory nitrate reductase in arbuscular mycorrhizal and other fungi. Mycorrhiza 5:23–28

Kaldorf M, Schmelzer E, Bothe H (1998) Expression of maize and fungal nitrate reductase genes in arbuscular mycorrhiza. Mol Plant-Microbe Interact 11:439–448

Lei J, Bécard G, Piché Y (1991) Root factors stimulate ^{32}P uptake and plasmalemma ATPase activity in the vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytol 118:289–294

Liberman DF, Wolfe L, Fink R, Gilman E (1996) Biological safety considerations for environmental release of transgenic organisms and plants. In: Levin MA, Israeli E (eds) Engineered organisms in environmental settings. CRC Press, Boca Raton, Florida, pp 42–63

Moriau L, Bogaerts P, Jonniaux JL, Boutry M (1993) Identification and characterization of a second plasma membrane H^+-ATPase gene subfamily in *Nicotiana plumbaginifolia*. Plant Mol Biol 21:955–963

Murray M, Thompson WF (1980) Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res 8:4321–4325

Nielsen KM, Bones AM, Smalla K, van Elsas JD (1998) Horizontal gene transfer from transgenic plants to terrestrial bacteria – a rare event? FEMS Microbiol Rev 22:79–103

Palmgren MG, Axelsen KB (1998) Evolution of P-type ATPases. Biochim Biophys Acta 1365:37–45

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85:2444–2448

Pérez C, Michelet B, Ferrant V, Bogaerts P, Boutry M (1992) Differential expression within a three-gene subfamily encoding a plasma membrane H^+-ATPase in *Nicotiana plumbaginifolia*. J Biol Chem 267:1204–1211

Pirozynski K, Dalpé Y (1989) Geologic history of the Glomaceae with particular reference to mycorrhizal symbiosis. Symbiosis 7:1–29

Pirozynski KA, Hawksworth DL (1988) Coevolution of fungi with plants and animals. Academic Press, London

Rao R, Slayman CW (1996) Plasma-membrane and related ATPases. In: Brambl R, Marzluf GA (eds) The Mycota III. Biochemistry and molecular biology. Springer-Verlag, Berlin, Heidelberg, pp 29–56

Redecker D, Thierfelder H, Walker C, Werner D (1997) Restriction analysis of PCR-amplified internal transcribed spacers of ribosomal DNA as a tool for species identification in different genera of the order Glomales. Appl Environ Microbiol 63:1756–1761

Remy W, Taylor TN, Hass H, Kerp H (1994) Four-hundred-million-year-old vesicular arbuscular mycorrhizae. Proc Natl Acad Sci USA 91:11841–11843

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Schelesser A, Ulaszewski S, Ghislain M, Goffeau A (1988) A second transport ATPase gene in *Saccharomyces cerevisiae*. J Biol Chem 263:19480–19487

Serrano R (1984) Plasma membrane ATPase of fungi and plants as a novel type of proton pump. Curr Top Cell Regul 23:87–126

Serrano R (1989) Structure and function of plasma membrane ATPase. Annu Rev Plant Physiol Plant Mol Biol 40:61–94

Serrano R, Kielland-Brandt MC, Fink GR (1986) Yeast plasma membrane ATPase is essential for growth and has homology with (Na^+ + K^+), K^+ and Ca^{2+}-ATPases. Nature 319:689–693

Simon L, Bousquet RC, Levesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plants. Nature 363:4211–4125

Smith FA, Raven JA (1979) Intracellular pH and its regulation. Annu Rev Plant Physiol 30:289–311

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Struck C, Siebels C, Rommel O, Wernitz M, Hahn M (1998) The plasma membrane H^+-ATPase from the biotrophic rust fungus *Uromyces fabae*: molecular characterization of the gene (PMA1) and functional expression of the enzyme in yeast. Mol Plant-Microb Interact 11:458–465

Sussman MR (1994) Molecular analysis of proteins in the plasma membrane. Annu Rev Plant Physiol Plant Mol Biol 45:211–234

Tanner W, Caspari T (1996) Membrane transport carriers. Annu Rev Plant Physiol Plant Mol Biol 47:595–626

Taylor TN, Remy W, Hass H, Kerp H (1995) Fossil arbuscular mycorrhizae from Early Devonian. Mycologia 87:560–573

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680