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Molecular approaches to study plasma membrane H^+-ATPases in arbuscular mycorrhizas

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Abstract

The activity of H^+-ATPases of plant and fungi 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. During the last years, several studies have been aimed at elucidating the role of plasma membrane H^+-ATPases in the nutrient exchange processes taking place between the plant and the fungus in arbuscular mycorrhizal (AM) symbiosis. This paper reviews present knowledge about plasma membrane H^+-ATPases and experimental evidence supporting the involvement of H^+-ATPases of both organisms in the bidirectional transport of nutrients between partners. Molecular strategies that will provide further information on the function and regulation of plasma membrane H^+-ATPases in AM symbiosis are presented and discussed.

Introduction

The benefit of arbuscular mycorrhizal (AM) symbiosis to plant development depends largely on the ability of the fungal mycelium to take up nutrients, in particular phosphate, from the soil solution and to transfer them to the symbiotic roots in exchange for carbohydrates (Azcón-Aguilar and Bago, 1994; Jakobsen, 1999). Although transport processes between plant and fungal cells are crucial for proper functioning of AM symbiosis, they represent one of the most poorly understood aspects of the symbiosis. Mechanisms for controlled transport of nutrients between the mycorrhizal symbionts are largely hypothetical and have been recently discussed in detail by Smith and Smith (1996) and Smith and Read (1997). Although not experimentally proved, it is accepted today that the bidirectional transfer of nutrients across the symbiotic interfaces involves passive efflux of solutes from the donor organism into the interfacial apoplast, followed by active uptake by the receiver organism (Gianinazzi-Pearson et al., 1991; Smith and Read, 1997; Woolhouse, 1975). However, there are still many unanswered questions, including the precise location at which nutrient transfer occurs and the molecular forms and mechanisms by which phosphate, and other mineral nutrients, and carbon compounds move across the individual membranes. Elucidation of these mechanisms requires identification of the specific transport proteins involved in efflux and uptake of solutes by both partners in the symbiotic interfaces. Since membranes involved in active uptake processes must be energized by a plasma membrane H^+-ATPase (Serrano, 1985), study of location and regulation of plasma membrane H^+-ATPases in AM symbiosis will also provide insights into the mechanisms involved in the controlled transfer of nutrients between symbionts. This paper summarizes present knowledge about plasma membrane H^+-ATPases, experimental evidence supporting the hypothesis that transport processes in AM symbiosis are driven by the plasma membrane H^+-ATPases of both symbionts, and new experimental approaches that will provide insights into the role of these enzymes in the symbiosis.

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Plasma membrane H^+-ATPases: an overview

Plasma membrane H^+-ATPases of plants and fungi are composed of a single catalytic polypeptide with an approximate molecular weight of 100 kDa that is phosphorylated and dephosphorylated during its catalytic cycle (Briskin and Hanson, 1992; Serrano, 1989). This property has led to the classification of this enzyme as a P-type ATPase. This protein transports one H^+ per molecule of ATP hydrolyzed, has a low K_m for ATP (0.2–2 mM) and its activity is inhibited by vanadate, diethylcarbodiimide and erythrosin B.

The plasma membrane H^+-ATPase is the enzyme primarily responsible for active transport in plants and fungi (Serrano, 1985). The activity of the H^+-ATPase 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 (Fig. 1) (Michelet and Boutry, 1995; Serrano, 1989; Sussman and Harper, 1989). Depending on the electric charge of the solute to be transported, the direction of its transport and its concentration on either side of the membrane, it is possible to predict from Fig. 1 the type of transport protein required. The uptake of a cation or the efflux of an anion is often energetically favourable because of the inside-negative electrical potential generated by the H^+-ATPase, and therefore requires only a diffusion facilitator, such as a channel protein or uniport. However, the uptake of a cation against its concentration gradient requires a H^+-cation symport system. To be energetically favourable, the uptake of an anion must usually be accompanied by the uptake of one or more H^+ in a symport system. The uptake of neutral molecules, such as carbon compounds or amino acids, is energetically unfavourable when it takes place against a concentration gradient and here, again, the proton motive force created by the plasma membrane H^+-ATPase provides the energy required for this transport.

In addition to activating secondary transport, the activity of H^+-ATPases also contributes to the maintenance of intracellular and extracellular pH (Smith and Raven, 1979), and it has been proposed that regulation of plasma membrane H^+-ATPases may mediate a broad range of physiological responses which play a central role in growth and development of higher plants and fungi (Serrano, 1989).

H^+-ATPase activity seems to be regulated by an extraordinary variety of mechanisms (reviewed by Michelet and Boutry, 1995; Rao and Slayman, 1996; Sussman, 1994). For instance, it has been shown that transcriptional regulation of the H^+-ATPase genes accounts for the tissue specific distribution of this enzyme in plants (Harper et al., 1994; Houlné and Boutry, 1994) and for the glucose activation of ATPases in yeasts (Rao et al., 1993). Moreover, regulation of the activity has also been shown to occur at several posttranscriptional steps, namely translation, protein targeting, protein stability and enzyme modulation via the C-terminal region and possibly (de)phosphorylation (Morsomme et al., 1996; Portillo et al., 1989; Regenber et al., 1995; Xing et al., 1996).

Molecular studies of plant and fungal H^+-ATPases have shown that there are several genes coding for H^+-ATPases. Up to now seven members have been identified in tomato (Ewing and Bennett, 1994), ten in *Arabidopsis* (Harper et al., 1994), four in tobacco (Moriau et al., 1993; Perez et al., 1992), two in rice (Ookura et al., 1994; Wada et al., 1992), two in *Saccharomyces cerevisiae* (Schlesser et al., 1988; Serrano et al., 1986) and two in *Schizosaccharomyces pombe* (Ghislain and Goffeau, 1991; Ghislain et al., 1987). The different isozymes may differ in their biochemical or regulatory characteristics, providing pools of functionally distinct H^+ pumps. The existence of multiple isoenzymes is certainly the major factor that allows the fine regulation of H^+-ATPases in different cells and tissues and in response to environmental stimuli. Sequence comparison of genes coding for these enzymes indicates a high degree of sequence similarity between each other (Serrano, 1989). The homology between the different isoforms of a species is extremely high. Biochemical characterization of different H^+-ATPase isoforms heterologously expressed in yeasts suggests that the small differences in amino acid sequences between the different isozymes are important for generating H^+ pumps with unique catalytic properties (Palmgren and Christensen, 1994).

Cytochemical and biochemical studies of plasma membrane H^+-ATPases in AM symbiosis

Cytochemical studies of ATPases based on the use of lead as a capture agent for phosphate released from hydrolysis of ATP by the ATPase were carried out by Gianinazzi-Pearson et al. (1991). These studies have shown the presence of high ATPase activity in the periarbuscular membrane of the plant, in the plasma membrane of the extraradical and intercellular hyphae,

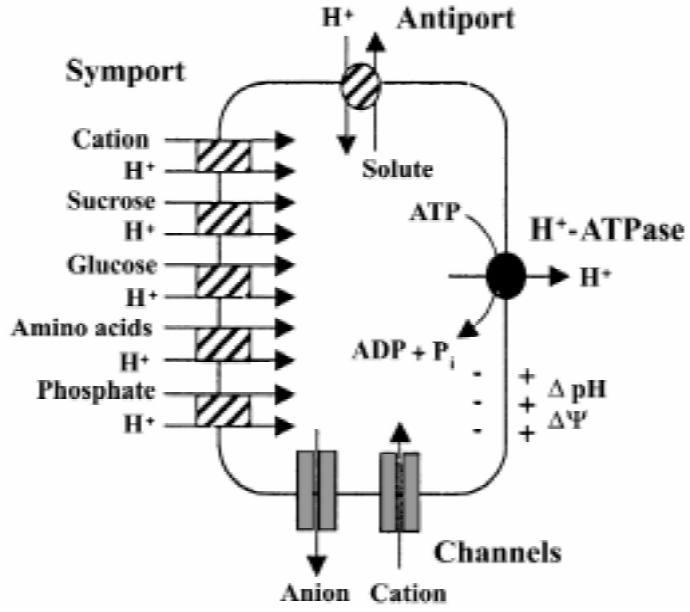

Figure 1. Schematic diagram of secondary transporters energized by the plasma membrane H^+-ATPase.

and also in the arbuscular trunk. However, the activity in plasma membranes of cells adjacent to the intercellular hyphae and in the fine hyphal branches of the arbuscules was weaker or absent. It was suggested that these ATPases represent H^+ pumps that establish H^+ motive forces and drive the uptake of P_i or hexose against their electrochemical gradients, from the interfacial apoplast into the root or fungal cells. The detection of ATPase activity on the plasma membrane of the intercellular hyphae, together with its absence in the fine arbuscular branches, led these authors to hypothesize that intercellular hyphae could also have a role in carbon uptake from the host apoplast, suggesting a certain spatial separation of P_i and C transfer (Gianinazzi-Pearson et al., 1991). However, no experimental support for this hypothesis has been yet provided.

The presence of ATPase activity on the plasma membrane of the extraradical hyphae suggests that uptake of nutrients from the soil solution is also carried out by active transport processes. The recent molecular cloning of a high-affinity phosphate transporter from the AM fungus *Glomus versiforme*, which is operated by a H^+-coupled symport and whose expression was located in the external hyphae, confirms this hypothesis (Harrison and van Buuren, 1995).

During the last few years, several biochemical studies using membrane vesicles isolated from mycorrhizal and non-mycorrhizal plants have been also aimed at investigating plasma membrane H^+-ATPases in AM symbiosis (Bago et al., 1997; Benabdellah et al., 1999; McArthur and Knowles, 1993). Studies of ATPases in isolated membrane vesicles have the advantage of allowing measurement in a single preparation of both the enzyme activity and the generation of the H^+ gradient. These studies have shown increases in plasma membrane ATPase activity of root microsomes isolated from mycorrhizal plants of different species (Bago et al., 1997). Recently, it has been demonstrated that membranes isolated from mycorrhizal roots showed an increased ATP-dependent H^+ pumping activity compared with membranes isolated from non-mycorrhizal roots, while the phosphohydrolytic activity of the H^+-ATPase was not affected (Benabdellah et al., 1999). Based on these data it has been suggested that in mycorrhizal plants plasma membrane ATPase could be specifically regulated by increasing the coupling efficiency between H^+ pumping and ATP hydrolysis. However, the mechanisms involved in this regulation are unknown. Molecular studies of ATPases in AM symbiosis are necessary to determine whether a specific isozyme with a higher H^+ pumping activity is induced by mycorrhizal colonization in tomato roots. Alternatively, the higher H^+ pumping activity observed in mycorrhizal roots could be the result of posttranslational modifications involving phosphorylation/dephosphorylation reactions.

Molecular approaches to study H^+-ATPases in AM symbiosis

Study of plasma membrane H^+-ATPase gene expression in AM symbiosis requires development of fungal and plant specific gene probes. This has been relatively easy for the plant genes because, as indicated above, the genes coding for the plasma membrane H^+-ATPases of several plants have already been isolated and characterized. However, molecular studies of the AM fungal H^+-ATPases require identification of the genes coding for this enzyme, which has been recently achieved by a PCR-cloning approach, as will be explained below. Once the nucleotide sequences coding for the different isozymes of the H^+-ATPases from both partners are known, information on the function and regulation of each gene can be obtained by expression studies of the target sequence.

The most common methods for gene expression studies are Northern blotting (the method involves mRNA isolation and assessment of hybridization with specific gene probes) and in situ hybridization (visualizing the site of gene expression in micrographs). However, Northern blot analyses lack the sensitivity needed for investigation of RNAs present in low abundance and the specificity required to estimate the contribution to overall expression of individual gene members of a multigene family. Accordingly, the study of H^+-ATPase gene expression (encoded by multigene families) in AM symbiosis by Northern blot can be limited, especially when looking at the expression of the fungal genes.

The adaptation of PCR methodology to the investigation of RNA has given researchers a method featuring speed, efficiency, specificity and sensitivity. Since RNA cannot be used as a template for PCR, RNA is converted into a complementary DNA (cDNA) suitable for PCR by reverse transcription. Then, the target cDNA is amplified by PCR using gene specific primers. The combination of both techniques is referred to as RT-PCR (Kawasaki, 1990). Although RT-PCR is a simple and rapid technique to detect mRNA transcripts, quantification of the amount of mRNA present in the starting material is more difficult because of the exponential nature of the PCR. Quantification of mRNA species by RT-PCR can be achieved by competitive (Gilliland et al., 1990) or semiquantitative (Dallman and Porter, 1991) PCR. This technique has been successfully applied to quantify mRNA levels of the different H^+-ATPase genes identified in tomato (Ewing and Bennett, 1994; Mito et al., 1996).

In vivo regulation of host H^+-ATPase genes by AM colonization can be also assessed using transgenic plants transformed with fusions of the presumed transcription promoter region of individual H^+-ATPase genes to a reporter gene (Michelet et al., 1994).

Molecular studies of host plasma membrane H^+-ATPases

The first study showing induction of a plant H^+-ATPase gene by AM colonization was reported by Murphy et al. (1997). By differential screening of a cDNA library prepared from highly AM colonized pieces of barley roots, these authors observed that one of the differentially expressed cDNA clones represented a partial cDNA encoding a plasma membrane H^+-ATPase. This gene was up-regulated in mycorrhizal roots but its expression level was unaffected by the addition of phosphate.

As indicated above, plant H^+-ATPases are encoded by multigene families. Considering that individual H^+-ATPase isoforms might possess unique functions and that expression of each gene may be differentially regulated by distinct physiological stimuli, understanding the physiological role of plasma membrane H^+-ATPases in AM symbiosis requires expression analysis of each gene family member. To study regulation of plasma membrane H^+-ATPases by AM symbiosis in tomato, whose gene family is composed of at least seven members, RT-PCR assays using isoform-specific primers have been carried out. Quantification of mRNA levels of three individual H^+-ATPase isozymes in mycorrhizal and non-mycorrhizal tomato roots has shown that expression of two isoforms was differentially regulated in AM roots (Ferrol and Azcón-Aguilar, unpublished results). Further research on the expression level of the other isoforms and on the localization of each H^+-ATPase gene in mycorrhizal roots is necessary to assess the potential role of specific isoforms in AM symbiosis. Recently, promoter-GUS fusion assays in transgenic tobacco plants have provided evidence that at least two plant plasma membrane H^+-ATPases are activated in arbuscule-containing cells (Gianinazzi-Pearson et al., 1998). These data suggest that regulation of host ATPases by AM symbiosis is a complex process. As indicated in the first section of this paper, the plasma membrane H^+-ATPase is a highly regulated enzyme with multiple physiological functions (Serrano, 1989). Considering that individual H^+-ATPase

isoforms might possess unique functions, activation of at least two isoforms in mycorrhizal roots of two plant species suggests that in AM symbiosis H^+-ATPases could participate in processes other than energization of plasma membranes involved in active transport processes in the symbiotic interfaces.

Molecular studies of fungal plasma membrane H^+-ATPases

Genes coding for plasma membrane H^+-ATPases in *Glomus mosseae* have been recently cloned by using a PCR-based approach (Ferrol et al., 1998). PCR-cloning approaches using highly degenerate primers are very useful in identifying heterologous genes by starting with minute amounts of DNA (Lee and Caskey, 1990), and therefore for the genetic characterization of unculturable organisms as AM fungi. A prerequisite for this approach is the identification of two conserved domains of about six amino acids in the cloned sequences of the target gene in other organisms, in order to be able to design the degenerate primers by back-translation of the amino acid sequences. Cloning of genes coding for plasma membrane H^+-ATPases in *G. mosseae* has been achieved by nested PCR of genomic DNA using two sets of degenerate primers. This approach significantly increases the sensitivity and specificity of the PCR since two pairs of primers are required to amplify the target sequence. The two sets of primers used for PCR amplification of *G. mosseae* genomic DNA were designed to match four highly conserved domains found at the catalytic site contained within the large hydrophilic loop present in all plant and fungal plasma membrane H^+-ATPases. The genomic DNA used for the PCR amplification was extracted from isolated and washed spores of *G. mosseae* (Ferrol et al., submitted).

Sequence analysis of the cloned PCR products identified five different clones (GmHA1, GmHA2, GmHA3, GmHA4 and GmHA5) coding for plasma membrane H^+-ATPases. These data indicate that in mycorrhizal fungi, as in other organisms, the plasma membrane H^+-ATPase is encoded by a multigene family. Southern blot hybridization of *G. mosseae* genomic DNA to each PCR clone (GmHA1 to GmHA5) demonstrated that each of the five cloned genes represented a gene from *G. mosseae* and not a gene from trace materials contaminating the spores. Analysis of the nucleotide and deduced amino acid sequences of the GmHA genes indicated that three of them show close similarity, while the other two are more divergent. Four of the cloned genes (GmHA1–GmHA4) showed the best homology to plasma membrane H^+-ATPases of other fungi and lower homology to plant ATPase genes. However, GmHA5 displayed about the same level of homology to plasma membrane H^+-ATPases of plants and fungi. This is in contrast from what would be predicted from taxonomic criteria, since within each phylogenetic kingdom ATPases show more than 75% homology. Although the evolutionary significance of the divergence of GmHA5 has to be elucidated, it could be the result of the intimate association and long period of co-evolution between AM fungi and plants.

The sequence differences in amino acids between the different plasma membrane H^+-ATPase isozymes in *G. mosseae* may be important for generating H^+ pumps with unique catalytic properties tailored to specific transport fiunctions. It is likely that members of the plasma membrane H^+-ATPase gene family of *G. mosseae* would be differentially expressed in the different fungal structures (spore germ tubes, appressoria, intra- or extra-radical hyphae, arbuscules, etc.) depending on the developmental and physiological stage of the symbiosis. Expression studies of the individual genes will provide insights into the physiological role of each plasma membrane H^+-ATPase isozyme in AM fungi.

Conclusions and perspectives

The results obtained using different approaches clearly show that both processes, nutrient uptake by the extraradical mycelium of AM fungi and nutrient exchange in the symbiotic interfaces, are carried out by active transport processes driven by the plasma membrane H^+-ATPase. Recently, efficient molecular biology strategies have been developed to study plasma membrane H^+-ATPases in AM symbiosis. Although these studies have just started, the molecular tools to study plant and AM fungal plasma membrane H^+-ATPases are now available. In situ hybridization and RT-PCR experiments will be aimed at assessing the spatial and temporal expression of the different isozymes of the plasma membrane H^+-ATPases of both partners. Results derived from these experiments will provide insights into the fungal and plant membranes involved in active transport processes in AM symbiosis. Moreover, these studies will allow to elucidate the physiological role of the different plasma

membrane H^+-ATPase isozymes during the development of AM symbiosis.

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