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Molecular Biology of the Plasma Membrane of Higher Plants

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REVIEW

INTRODUCTION

In plants, as in all organisms, the plasma membrane serves two main roles: (1) the transport of solutes, both into and out of each cell, and (2) sensory transduction, i.e., the sensing and initiation of the cellular response to changing environmental conditions. Recently, considerable progress has been made in elucidating the role of specific plasma membrane proteins in these two processes. Historically, in all eukaryotes, this research area was hampered by problems in membrane purification as well as by difficulties in the purification and sequencing of water-insoluble membrane proteins. Increased ease in DNA cloning and sequencing procedures, as well as improvements in detergent-based chromatography techniques, have alleviated some of these problems. Thus, although the plasma membrane has been the subject of physiological and biochemical research for several decades, only recently has the molecular analysis of plasma membrane proteins been achieved.

This past year the first genes encoding plant plasma membrane proteins were cloned and sequenced. In reports from two separate laboratories, three genes encoding plasma membrane proton pumps (H^+-ATPases) from *Arabidopsis thaliana* were described (Harper, Surowy, and Sussman, 1989; Pardo and Serrano, 1989), and a third laboratory has recently described genes encoding this enzyme from tobacco (*Nicotiana plumbaginifolia*) (Boutry, Michelet, and Goffeau, 1989). As of this writing there are two other preliminary reports on cloning plasma membrane protein genes from higher plants: ATPase genes from tomato (Bennett, Ewing, and Wimmers, 1989) and G-protein genes from corn (Palme et al., 1989). This review will focus on recent findings concerning the genomic and cDNA structure of genes encoding the plasma membrane proton pump from higher plants and relate them to pumps from other organisms. In addition, we will present a list of other known plant plasma membrane proteins and discuss future strategies for cloning their genes. For additional general reviews on the plasma membrane proton pump, see Sussman and Surowy (1987), Serrano (1988a, 1988b, 1989), Rao, Nakamoto, and Slayman (1989), Nakamoto and Slayman (1989), and Nakamoto, Rao, and Slayman (1989).

Plasma membrane research is aided by the availability of electrophysiological techniques that complement biochemical and genetic procedures for studying protein function. Unlike microbial cells, some plant cells are sufficiently large to permit the insertion of microelectrodes (Felle, 1982), thus allowing in situ measurements of ion pumps and other transporters. In plant research this has proven especially useful for interpreting biochemical results because plasma membrane transport proteins become labile due to the action of the proteases and lipases that are inevitably released from the large central vacuole during homogenization (Gallagher, Carroll, and Leonard, 1986; Gallagher and Leonard, 1987).

For this discussion it is useful to clarify some electrophysiological terms. Transport proteins are generally grouped into three broad classes: pumps, carriers, and channels. These classifications are based on different forms of energy input: chemical bond energy (i.e., ATP) or light energy for pumps and the energy of electrochemical ion gradients for carriers (symport or antiport) and channels (uniport). Another basis for these categories is the speed of transport, i.e., the number of solute molecules transported per protein per second, with pumps relatively slow (less than 500 per second), carriers intermediate (500 to 10,000 per second), and channels the most rapid (10,000 to many million per second).

An important concept that has emerged from plasma membrane research over the past decade is the primary role of two cation pumps, the proton pump (H^+-ATPase) of plant and fungal cells and the sodium pump (Na^+,K^+-ATPase) of animal cells. These two enzymes play pivotal roles in the nutrition of eukaryotes because they "energize" the membrane by generating the motive force that drives solute carriers and channels. In accord with the ion specificity of the two primary cation pumps, the carriers in animals are mostly sodium-coupled, whereas those in

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plants and fungi are proton-coupled. In plants and fungi, the primary cation pump transports a single proton for every ATP hydrolyzed (Perlin et al., 1986; Brauer et al., 1989) and thereby generates a large electric potential (as high as −300 mV, internal negative) and a smaller proton chemical gradient (about pH 7 inside and pH 5 outside). Importance of the plasma membrane proton pump is underscored by electrophysiological measurements that indicate that in actively transporting cells, such as *Neurospora crassa* hyphae and root hairs of higher plants, the plasma membrane proton pump consumes as much as ¼ to ½ of the cellular ATP (see Felle, 1982, and discussion in review by Goffeau and Slayman, 1981).

The feasibility of purifying transport proteins is dependent on their abundance. Channel proteins are often present at low concentrations, and as few as 10 channel proteins per cell may be sufficient to regulate cytoplasmic levels of an ion. At the other extreme, in actively transporting cells, such as rapidly growing fungi, the proton pump may be the major polypeptide in the plasma membranes, accounting for 5% to 10% of the total membrane protein (Bowman, Blasco, and Slayman, 1981). In plasma membrane vesicles extracted from roots of higher plants, the proton pump accounts for, at most, only 1% of the membrane protein and 0.01% of the total cellular protein, even though it is still one of the more abundant polypeptides in the plasma membrane (Schaller and Sussman, 1988b). Thus, with most pumps, a biochemical approach is feasible for characterizing their protein structure, whereas with channels, a genetic approach may be the only practical means to obtain this information.

PLASMA MEMBRANE ATPase GENES

Biochemical and electrophysiological techniques have established that the major ATPase activity associated with the plasma membrane of plant and fungal cells is a proton pump (H^+-ATPase) (cf., Goffeau and Slayman, 1981; Serrano, 1985). There are three types of proton-pumping ATPases found in nature (Bowman and Bowman, 1986): (1) the F_0F_1 H^+-ATPase observed in mitochondria, chloroplasts, and bacteria that contains at least nine different polypeptides; (2) the vacuolar/lysosomal H^+-ATPase that contains at least three different polypeptides; and (3) "P-type" ATPases, so called because they form a stable phosphorylated intermediate at an aspartic acid residue. Most members of this last group (the H^+-, H^+,K^+-, and Ca^{2+}-ATPases) consist of a single catalytic polypeptide of about M_r = 100,000 with two exceptions, the Na^+,K^+-ATPase and the K^+-ATPase, which contain additional polypeptides of lower molecular weight. This group has also been called the "E_1E_2"-type because they all show at least two distinct conformational states, designated E_1 and E_2, during the reaction cycle.

Functional and structural studies with the purified enzyme and amino acid sequence data all indicate that the plant plasma membrane proton pump belongs to the P-type class of proton pumps (Schaller and Sussman, 1988b). Other well-characterized members of this group whose genes have been cloned and sequenced include (1) the fungal plasma membrane proton pump (H^+-ATPase) (Hager et al., 1986; Serrano, Kielland-Brandt, and Fink, 1986) as well as a putative proton pump in the plasma membrane of the protozoan, *Leishmania donovani* (Meade et al., 1989); (2) the Na^+,K^+-ATPase in mammalian plasma membrane (Shull, Schwartz, and Lingrell, 1985; Shull and Lingrel, 1987); (3) the K^+-ATPase of bacterial plasma membranes (Walderhaug, Litwack, and Epstein, 1989); (4) an H^+,K^+-ATPase in mammalian stomach (Shull and Lingrel, 1986; Maeda, Ishizaki, and Futai, 1988); (5) the Ca^{2+}-ATPase of mammalian plasma membrane (Shull and Greeb, 1988; Verma et al., 1988); and (6) the Ca^{2+}-ATPase of mammalian sarcoplasmic and endoplasmic reticulum (MacLennan et al., 1985; Brandl et al., 1986; Lytton and MacLennan, 1988; Lytton et al., 1989).

Biochemical analysis of the plant plasma membrane proton pump has proven difficult because of its lability during purification and reduced abundance relative to the fungi and other better characterized systems. Because these ATPases share a high degree of amino acid sequence identity in several regions of the polypeptide, a reasonable alternative to biochemical characterization is to exploit these identities to exhaustively clone and sequence all of the genomic and cDNA P-type ATPase clones from one plant species. Recent studies utilizing this approach in our laboratory at the University of Wisconsin in Madison, Wisconsin and that of R. Serrano at the European Molecular Biology Laboratory in Heidelberg, Federal Republic of Germany have identified three P-type ATPase genes in *A. thaliana*. We have given them trivial abbreviations of AHA1, AHA2, and AHA3 (for ***A**rabidopsis* **H**$^+$-**A**TPase). The complete cDNA sequence for AHA1 is reported in Harper, Surowy, and Sussman (1989) and the complete cDNA and genomic sequence of AHA3 is in Pardo and Serrano (1989). The genomic sequence and intron positions in AHA2 have also recently been determined in our laboratory, allowing a comparison of the three protein structures predicted from DNA sequence.

Two lines of evidence suggest that the three *Arabidopsis* P-type ATPase genes encode H^+ pumps located within the plasma membrane. First, the three all show greatest overall amino acid sequence identity (36%) with the fungal plasma membrane H^+-ATPases rather than the Ca^{2+}- (27%), H^+,K^+- (24%), Na^+,K^+- (22%), and K^+- (21%) ATPases included in the P-type class. Second, there is greater than 90% identity in amino acid sequence between that predicted by the clones and that determined by Edman degradation of random tryptic peptides derived from the ATPase of oat root plasma membranes. Electrophysiological and biochemical studies from several laboratories over

the past decade have demonstrated that the predominant ATPase activity in this preparation is a H^+ pump (H^+-ATPase). Although indirect, these observations suggest that the three genes cloned from *Arabidopsis* encode the plasma membrane proton pump.

It is clear from the predicted amino acid sequence that all three proteins are very similar. All encode a 949-amino-acid-long polypeptide, with a molecular weight of about 104,000. AHA1 and AHA2 are more closely related to each other than either is to AHA3: AHA1 and AHA2 show 95% amino acid identity, whereas AHA1 and AHA3 are 88% identical and AHA2 and AHA3 are 87% identical. AHA3 contains 15 introns within the protein coding region, and the first intron, located 15 amino acids away from the N-terminal methionine, is 1010 bp, the largest yet reported to occur in *Arabidopsis*. The other 14 introns are much smaller, varying from 69 bp to 222 bp. A large intron is also found in the same location in AHA2. Intron locations have not yet been determined for AHA1. Based on a similarity of AHA3 intron locations with intron locations for genes encoding other P-type animal Na^+,K^+- and Ca^{2+}-ATPases, it has been suggested that the AHA3 exon boundaries may correspond to structural modules within this family of enzymes (Pardo and Serrano, 1989).

A computer-generated hydropathy plot for the *Arabidopsis* plasma membrane H^+-ATPase protein sequence predicts a structure for the plant enzyme that is similar to the common motif of P-type ATPases (Harper, Surowy, and Sussman, 1989). This analysis denotes transmembrane segments as any stretch of 20 to 30 amino acids that contains a preponderance of hydrophobic amino acids and has successfully predicted a structure for the photosynthetic reaction center that is confirmed by high-resolution x-ray crystallographic data (Engelman, Steitz, and Goldman, 1986).

For this discussion it is useful to compare a plot derived for the *Arabidopsis* plasma membrane H^+-ATPase sequence with a very similar plot obtained with the *Neurospora* plasma membrane H^+-ATPase (see Figure 4 in Harper, Surowy, and Sussman, 1989). Although high-resolution x-ray crystallographic data are not yet available for the *Neurospora* enzyme, immunological and proteolytic studies (Mandala and Slayman, 1989) have confirmed the general features of this model. As illustrated in Figure 1, these features include: (1) 20% of the protein is embedded in the membrane and is distributed among eight transmembrane segments, (2) 70% is hydrophilic and exposed on the cytoplasmic surface, and (3) only 5% is exposed to the extracellular surface. The cytoplasmic portion is mainly found in two large loops, one between transmembrane segments 2 and 3 and a second located between transmembrane segments 4 and 5.

The hydropathy plot for all of the P-type ATPases is similar, and offers a straightforward interpretation for the enzyme topology within the N-terminal and central portions. However, there is considerable confusion in the analysis of carboxy portions (Davis and Hammes, 1989). For example, from a comparison of the hydropathy plots, it is apparent that there may exist one or two additional transmembrane segments in the plant enzyme that are not evident in the *Neurospora* enzyme (Harper, Surowy, and Sussman, 1989). More direct chemical or genetic (Manoil and Beckwith, 1986) studies will be needed to clarify this point.

An interesting difference between the plant and fungal enzymes is that the plant hydrophilic carboxy terminus appears considerably longer. Speculation based on two lines of evidence suggests that this carboxy terminus plays a regulatory role in controlling catalytic activity. First, in the plasma membrane Ca^{2+}-ATPase of both mammals (James et al., 1988; Shull and Greeb, 1988; Verma et al., 1988) and yeast (Rudolph et al., 1989), the carboxy termini contain a calmodulin-binding domain that has been implicated in regulating its activity via a Ca^{2+}-calmodulin transduction pathway. A second observation concerns the activation of the yeast plasma membrane H^+-ATPase by glucose (Serrano, 1983; Portillo and Mazon, 1986). This activation is observed within 30 sec of glucose addition to starved cells, indicating that a post-translational modification of the protein conformation may be involved. Recently, when a yeast mutant was constructed in which the last 11 amino acids at the carboxy terminus were deleted, the enzyme was observed to be constitutively activated, even in the absence of sugar (Portillo, de Larrisoa, and Serrano, 1989). This observation suggests that the carboxy terminus performs a regulatory role with the yeast plasma membrane H^+-ATPase. Activation of the plant enzyme has likewise been implicated as an early response in the signal transduction pathway for blue light (Assmann, Simoncini, and Schroeder, 1985) and hormones (Felle, 1982). It is tempting to speculate that the additional unique sequences found in the plant enzyme carboxy terminus play a direct role in these events.

Why are there three separate genes for this enzyme in *Arabidopsis*? Multiple plasma membrane H^+-ATPase genes have been observed in tobacco (Boutry, Michelet, and Goffeau, 1989) and tomato (Bennett, Ewing, and Wimmers, 1989), but it seems more surprising to find this multiplicity in *Arabidopsis*, which has the smallest genome size (about 70,000 kb) of any plant species (Meyerowitz and Pruitt, 1985). Because all three *Arabidopsis* genes were found in cDNA libraries, it is clear that all three genes are expressed. Higher plants are characterized by the presence of specialized and highly differentiated cell types with unique transport functions (e.g., root hair, phloem-loading, or guard cells), and we may speculate that the three *Arabidopsis* plasma membrane H^+-ATPase genes show differential levels of expression in these cells. Multiple genes are an obvious means for providing isoforms with unique properties and levels of expression to meet the specialized transport needs for a particular cell type. For Na^+,K^+-ATPase in mammals this is clearly the case,

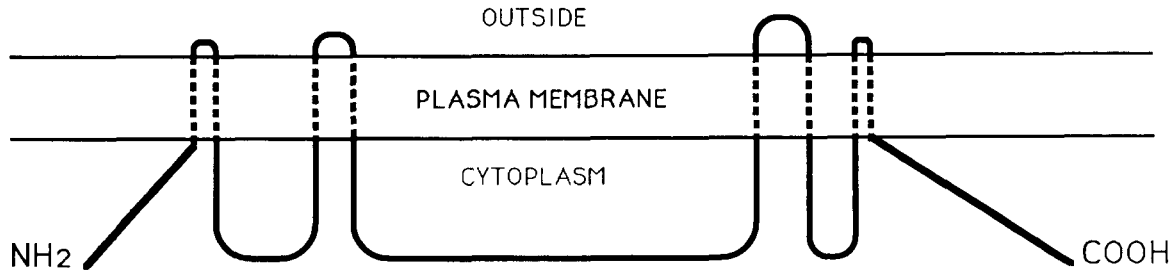

Figure 1. A Model for the Structure of the *A. thaliana* Plasma Membrane H^+-ATPase Protein, as Predicted from a Hydropathy Plot of the DNA-Derived Amino Acid Sequence (Harper, Surowy, and Sussman, 1989).

Transmembrane sequences are shown as dashed lines between the membrane surfaces.

with at least three separate Na^+,K^+-ATPase genes with various levels of expression in differentiated tissues (Orlowski and Lingrel, 1988; Schmitt and McDonough, 1988; Sverdlov et al., 1988, 1989). For example, in some kidney cells, a Na^+,K^+-ATPase isoform seems to account for 10% to 50% of the plasma membrane protein, whereas in other, less specialized mammalian tissue this enzyme is present at a 100-fold lower level of expression. Hormone activation of specific Na^+,K^+-ATPase genes has also been reported (Haber and Loeb, 1988).

In yeast there are two P-type plasma membrane H^+-ATPase genes that have been cloned, sequenced, and disrupted. The first is called *pma1*, and disruptions in its protein coding sequence are lethal, i.e., the haploid spores do not germinate (Serrano, Kielland-Brandt, and Fink, 1986). Nonlethal disruptions of the promoter sequence produce mutants with reduced levels of ATPase expression and reduced growth rates (see also Capieaux et al., 1989). The protein predicted from a clone of a second yeast P-type ATPase gene (*pma2*) shows extensive homology to *pma1*, suggesting a similarity in function as a proton pump (Schlesser et al., 1988). The observed normal growth of haploid strains carrying a disrupted *pma2* gene eliminates the possibility that *pma2* plays an essential role in cell growth, although other observations suggest that *pma2* contributes to generating the proton-motive force under some conditions. In conclusion, results from yeast, as in plants, do not yet point to a clear reason for multiplicity with genes encoding the plasma membrane proton pump.

FUTURE PROSPECTS

Obviously, the application of molecular biology techniques to studying plasma membrane function in higher plants is only now beginning. cDNA and genomic clones encoding the primary active pump, the proton pump, have been isolated and sequenced. Efforts are underway at utilizing these DNA clones to evaluate cellular, developmental, or hormone-specific expression of particular isoforms. Utilizing gene transformation procedures, the establishment of an expression system for these genes in higher plants will test current hypotheses concerning the role of the plasma membrane proton-motive force in growth and development, nutrient uptake, hormone action, and pathogenesis. The cloned ATPase genes can be used to generate a battery of monoclonal or polyclonal antibodies directed to specific domains within specific isoforms. Such antibodies will prove particularly useful in immunocytochemical studies of plasma membrane biogenesis and function. Genetic and biochemical experiments with the cloned genes can be devised to test for sequences essential for targeting the proton pump to the cell surface (Holcomb et al., 1988). Finally, an agriculturally oriented use for the cloned DNA will be to test whether any heritable differences in nutrient uptake are caused by variant plasma membrane ATPase genes using restriction fragment length polymorphism mapping techniques.

There are several other plant plasma membrane proteins for which the prospect of cloning their structural genes in the immediate future is promising. G proteins are ubiquitous polypeptide components in the sensory transduction pathway across the plasma membrane of all eukaryotes. Plant plasma membrane proteins with properties similar to G proteins were recently characterized (Blum et al., 1988), and cDNA clones encoding putative G proteins from corn have now been isolated and sequenced (Palme et al, 1989). These clones were obtained with synthetic DNA oligonucleotides directed to conserved amino acid sequences in G proteins isolated from nonplant sources. The corn sequence demonstrates that plants contain genes encoding these important proteins, and preliminary information suggests that these genes show tissue-dependent expression. It is interesting to note that, although the predicted amino acid sequence shows no transmembrane sequences, it displays a conserved domain surrounding a cysteine adjacent to the carboxy terminus. In other eukaryotes this cysteine may become palmitylated, thus anchoring the protein to the membrane.

Table 1 offers a list of plant plasma membrane proteins

Table 1. Strategies for Cloning Plasma Membrane Protein Genes from Higher Plants

Protein	Reference	Strategy
Receptor for *N*-1-naphthylphthalamic acid (auxin carrier)	Short and Jacobs (1987) Jacobs and Short (1987)	Purify protein[a]
Receptor for fusicoccin	de Boer, Watson, and Cleland (1989) Meyer, Feyerabend, and Weiler (1989)	Purify protein
Receptor for verapamil	Harvey, Venis, and Trewava (1989)	Purify protein
Receptor for indole-3-acetic acid (auxin)	Barbier-Brygoo et al. (1989) Hicks, Rayle, and Lomax (1989) Hicks et al. (1989) Jones and Venis (1989)	Purify protein *A. thaliana* mutants[b]
Sucrose carrier	Bush (1989) Gallet et al. (1989) Lemoine et al. (1989) Ripp et al. (1988)	Purify protein
Amino acid carrier	Bush and Langston-Unkefer (1989)	Purify protein *A. thaliana* mutants
Nitrate carrier	Doddema and Telkamp (1979)	*A. thaliana* mutants
(1,3)-β-Glucan synthase	Lawson et al. (1989)	Purify protein
Ca^{2+}-ATPase	Briars, Kessler, and Evans (1988) Briars and Evans (1989)	Purify protein Heterologous probing[c]
Protein kinases	Schaller and Sussman (1988a) Farmer, Pearce, and Ryan (1989) Ladror and Zielinski (1989)	Purify protein Heterologous probing
Mechanoreceptor ion channels	Falke et al. (1988)	Heterologous probing *A. thaliana* mutants cDNA injection in *Xenopus oocytes*[d]
Potassium channels and other ion channels	Hedrich and Schroeder (1989) Schroeder and Hedrich (1989) Tester (1989)	Heterologous probing *A. thaliana* mutants cDNA injection in *Xenopus oocytes*
b-Type cytochromes and other redox enzymes	Asard et al. (1989) Leong and Briggs (1981) Luster and Buckhout (1988)	Purify protein

[a] Purify protein, then make antibodies and/or generate amino acid sequence information via automated Edman degradation of the N terminus or of internal peptide fragments. Antibody can be used to isolate the genes by screening expression libraries (e.g., λgt11). DNA oligonucleotide probes are generated, based on the protein sequence, and used to screen plant DNA libraries or as primers in the polymerase chain reaction with plant DNA.

[b] Mutant *A. thaliana* plants can be selected for that contain lesions in this genetic locus. These mutants can, in theory, be used to isolate the DNA by either chromosomal walking (via restriction fragment length polymorphisms) or "tagging" via insertional mutagenesis with *Agrobacterium* T-DNA or transposons. For auxin receptor mutants, mutations that affect auxin sensitivity are suggested; for channel mutants, resistance to inhibitors such as gadolinium (mechanoreceptor) or cesium (potassium channel) are suggested; for amino acid carriers, resistance to amino acid analogues is suggested.

[c] Heterologous probing means using conserved amino acid sequence from nonplant species (e.g., yeast and mammals) to synthesize a synthetic DNA oligonucleotide that can be used as probe for screening plant DNA libraries, or as primers for the polymerase chain reaction with plant DNA.

[d] A common procedure to isolate mammalian plasma membrane ion channel genes is to inject fractionated cDNA subpopulations into *Xenopus* oocyte eggs and monitor, via the electrophysiological technique known as "patch-clamping," for a fraction that generates a foreign channel signal.

that have been characterized by biochemical or electrophysiological studies and for which we can expect to see cloned genes isolated in the near future. Excluded are proteins whose existence in the plant plasma membrane is uncertain. The list is subjective and certainly does not include plasma membrane proteins whose genes will be cloned by ingenious strategies yet to be devised. These membrane proteins are particularly difficult to characterize using conventional biochemical procedures. Molecular biology techniques circumvent some of the problems and should continue to have an expanding role in research on the plasma membrane of higher plants. Because the plasma membrane plays a central role in so many physiological processes of the plant, it is likely that the application

of genetic techniques in this area will have a large impact in many related fields of study, including the regulation of growth and development, hormone action, and resistance to pathogens and stress.

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