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Molecular cloning and sequence of cDNA encoding the plasma membrane proton pump (H^+-ATPase) of *Arabidopsis thaliana*

(cation pumps/nucleotide sequence/amino acid homology/oligonucleotide screening/transmembrane segments)

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ABSTRACT **In plants, the transport of solutes across the plasma membrane is driven by a proton pump (H^+-ATPase) that produces an electric potential and pH gradient. We have isolated and sequenced a full-length cDNA clone that encodes this enzyme in *Arabidopsis thaliana*. The protein predicted from its nucleotide sequence encodes 959 amino acids and has a molecular mass of 104,207 Da. The plant protein shows structural features common to a family of cation-translocating ATPases found in the plasma membrane of prokaryotic and eukaryotic cells, with the greatest overall identity in amino acid sequence (36%) to the H^+-ATPase observed in the plasma membrane of fungi. The structure predicted from a hydropathy plot contains at least eight transmembrane segments, with most of the protein (73%) extending into the cytoplasm and only 5% of the residues exposed on the external surface. Unique features of the plant enzyme include diverged sequences at the amino and carboxyl termini as well as greater hydrophilic character in three extracellular loops.**

The transport of soil nutrients into and within plants is an active process requiring an input of metabolic energy. Cellular metabolism is coupled to solute transport by means of the pH and electrical gradient generated by a proton pump (H^+-ATPase) embedded within the plasma membrane (1). In addition, an early event in the action of growth-modifying pathogens (2), hormones (3), and light (4) is an alteration in the plasma membrane proton pump activity. These observations have implied that the plasma membrane proton pump plays a direct role in the regulation of growth, perhaps by control of pH in the cytoplasm or cell wall (5).

On the basis of polypeptide composition and sensitivity to inhibitors, the plant plasma membrane H^+-ATPase is readily distinguished from proton pumps found in membranes derived from the chloroplast, mitochondria, and vacuole (6). The purified enzyme contains a single polypeptide of ≈100,000 Da that shows similarities in reaction mechanism and structure to a group of cation-pumping ATPases: the H^+-ATPase of fungal plasma membranes, the Na^+,K^+-ATPase of animal plasma membranes, the Ca^{2+}-ATPase of muscle sarcoplasmic reticulum, the H^+,K^+-ATPase of gastric mucosa plasma membrane, and the K^+-ATPase of *Escherichia coli* plasma membrane (7).

The plasma membrane proton pump has only been observed in plants and fungi and was characterized by biochemical and electrophysiological techniques (1, 8). Recent studies with the fungi *Neurospora crassa* and *Saccharomyces cerevisiae* have utilized molecular genetic techniques to investigate this enzyme's biological role and catalytic function (9, 10). To initiate a genetic analysis of this enzyme's function in higher plants, we sought to isolate plasma membrane H^+-ATPase genes from *Arabidopsis thaliana*. This plant was chosen because, with a short generation time and a small genome, it is most attractive for future detailed genetic studies (11). With protein sequence data derived from the plasma membrane proton pump of *Avena sativa* (oat), we first synthesized an oligonucleotide probe to screen an oat cDNA library and then used a purified oat clone to isolate a full-length *A. thaliana* cDNA clone. Here we provide evidence for the presence of at least two genes encoding plasma membrane proton pumps in *A. thaliana* and report the predicted amino acid sequence from one.

MATERIALS AND METHODS

Plants. *A. thaliana* L. cv. Columbia and *Avena sativa* L. cv. Gary (oat) were plant materials used in this study. Unless otherwise noted, standard molecular techniques were performed according to Maniatis *et al.* (12), and enzymes were purchased from New England Biolabs, Promega Biotec, or Pharmacia.

cDNA Cloning. A 26-residue, 16-fold degenerate oligonucleotide mixture was synthesized from phosphoramidites on an Applied Biosystems DNA Synthesizer at the University of Wisconsin Biotechnology Center. The 26-mer (denoted GDGV26I) has the following sequence: dGCITCITT(I/C)AC-(I/C)CCITC(I/C)CC(I/C)GTCAT-OH and encodes an antisense strand for the amino acid sequence Met-Thr-Gly-Asp-Gly-Val-Asn-Asp-Ala. Prior to use in screening, the oligonucleotide probe mixture was end-labeled with [γ-^{32}P]dATP (Amersham) to a specific activity of 1.1×10^9 dpm/μg with T4 polynucleotide kinase.

An *Avena sativa* cDNA library with G+C-tailed inserts harbored in pBR322 was generously provided by J. Lissemore and P. Quail (13). Colony hybridizations were performed at 30°C in 6× SSC (1× = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0)/1× Denhardt's solution (1× = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/0.05% (wt/vol) sodium pyrophosphate/yeast tRNA at 100 μg/ml/20% (vol/vol) formamide containing $1.1–2.2 \times 10^7$ dpm of ^{32}P-labeled GDGV26I per ml. Hybridized nitrocellulose filters were washed in 6× SSC/0.05% (wt/vol) sodium pyrophosphate for 1 hr at 30°C and 15 min at 35°C followed by exposure to Kodak-X-Omat AR x-ray film with Cronex intensifying screens at −70°C.

One positive oat cDNA clone containing a 1.85-kilobase (kb) insert (pTS1.85) was purified. An internal 0.6-kb *Sau*96I fragment was subcloned into the *Hin*cII site of the Promega vector pGEM 4Z (pTS.6). For use in probing an *A. thaliana* cDNA library, the 0.6-kb fragment of pTS.6 was excised by digestion with *Pst* I and *Bam*HI, gel purified, and labeled by nick-translation with [α-^{32}P]dCTP (Amersham) by using a commercial kit (Bethesda Research Laboratories).

A once-amplified *A. thaliana* cDNA library, constructed with λ ZAP (Stratagene) was generously provided by P. Hatfield and J. Callis (University of Wisconsin–Madison, Department of Horticulture). The library provided was amplified from 100,000 recombinants constructed by the method of Gubler and Hoffman (14) with poly(A)-enriched RNA

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extracted from young green leaves. cDNAs were cloned as *Eco*RI inserts. Plaque hybridizations and washes were performed at 55°C. Hybridizations were in 6× SSC/20 mM Tris·HCl (pH 7.6)/10 mM EDTA/0.5% (wt/vol) *N*-lauroylsarcosine/5× Denhardt's solution containing denatured and sonicated salmon sperm DNA at 100 μg/ml. The final high stringency wash was in 0.3× SSC/5 mM EDTA/0.2% (wt/vol) SDS. Three phage isolates were converted to plasmid clones according to the λ ZAP excision protocol provided by Stratagene. Clones pAHA1 and pAHA3 (*Arabidopsis* H^+-ATPase) were shown by restriction and sequence analysis to be identical and carry a fusion of two cDNAs encoded in 3.2-kb and 0.5-kb *Eco*RI fragments. The 3.2-kb *Eco*RI fragment was subcloned into the *Eco*RI site of pBluescript SK(+/−) and named pAHA1-11 (see Fig. 1). The third clone contained a 2.2-kb insert and was named pAHA2.

DNA Sequencing. Dideoxy sequencing (15) of pTS.6, pAHA1, and pAHA2 was performed using a Sequenase kit (United States Biochemical). Double-stranded DNA was used as a template and sequenced in both directions. Most of the sequencing reactions for pAHA1 and pAHA2 utilized specific oligonucleotide primers that were synthesized at the University of Wisconsin Biotechnology Center. The remaining sequence reactions utilized T7 (Promega Biotec) or T3 (New England Biolabs) primers with pAHA1, pAHA2, or derivative subclones as templates. The overall strategy for sequencing the 3.2-kb ATPase gene from pAHA1 is shown in Fig. 1. DNA sequences were analyzed using the University of Wisconsin Genetics Computer Group software on a VAX/VMS computer (16).

Southern Blot Analysis. Genomic DNA was extracted from 5-day-old *A. thaliana* seedlings germinated under sterile conditions, frozen in liquid nitrogen, and pulverized in a Braun coffee grinder. The DNA was then isolated by the cetyltrimethylammonium bromide precipitation and CsCl banding method of Murray and Thompson (17). Samples (4 μg) of genomic DNA were digested with a 5-fold excess of enzyme (relative to the recommendations of the suppliers), electrophoresed in 0.8% (wt/vol) agarose, transferred to a nylon membrane (MSI, Fisher), and hybridized and washed as described above for plaque hybridization, except at a higher incubation temperature (65°C).

RESULTS

Isolation and Sequence of a Full-Length *A. thaliana* cDNA Clone. To isolate a cDNA clone encoding the plant plasma membrane proton pump, we first synthesized a 26-mer oligonucleotide that is complementary to a peptide sequence, Met-Thr-Gly-Asp-Gly-Val-Asn-Asp-Ala, conserved in all known 100,000-Da eukaryotic cation-pumping ATPases and known to be present in tryptic peptides of the plasma membrane H^+-ATPase purified from *Avena sativa* (7). Deoxyinosine was incorporated in place of adenosine, thymidine, and guanosine in order to reduce the degeneracy warranted by unknown codon preference (18). By using an *Avena sativa* (oat) cDNA library enriched 5-fold for mRNA encoding >100,000-Da polypeptides, 100,000 colonies were screened with the oligonucleotide probe, and one positive clone containing a 1.85-kb insert (pTS1.85) was obtained. Confirmation that this insert encodes the plasma membrane H^+-ATPase was obtained by dideoxy DNA sequencing of a subclone (pTS.6) containing a 600-base-pair (bp) fragment encompassing the target oligonucleotide (data not shown). The predicted protein sequence encoded by this fragment showed 100% identity to a 16-amino acid long tryptic peptide derived from the purified oat enzyme (19) as well as extensive homology to fungal plasma membrane proton pumps.

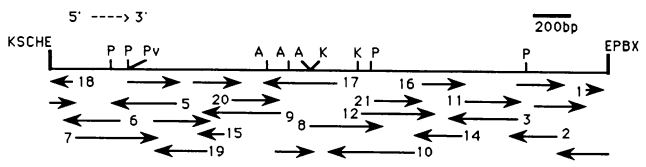

FIG. 1. Restriction map of pAHA1-11 and strategy for sequencing a cDNA encoding the *A. thaliana* plasma membrane H^+-ATPase. Arrows indicate sequence obtained from pAHA1-11 or derivative subclones with specific primers (indicated by numbers) or T7 and T3 primers (no numbers). The direction of transcription of the gene is indicated by 5′ to 3′. Restriction sites for selected enzymes are abbreviated as follows: A, *Ava* I; B, *Bam*HI; C, *Cla* I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn* I; P, *Pst* I; Pv, *Pvu* II; S, *Sal* I; X, *Xba* I. The *Eco*RI sites mark the insert boundaries. The vector is pBluescript SK(+/−).

Approximately 100,000 plaques of an *A. thaliana* cDNA phage library were screened with a 0.6-kb fragment that was gel-purified from the oat clone pTS.6. Two independent clones, pAHA1-11 and pAHA2, were obtained. The complete nucleotide sequence of pAHA1-11 was determined by using the sequencing strategy described in Fig. 1, and the predicted amino acid sequence is shown in Fig. 2. Computer analysis demonstrated that there is only one uninterrupted open reading frame encoding a protein with 959 amino acid residues and a molecular mass of 104,207 Da. The predicted ATG start codon occurs 90 nucleotides from the *Eco*RI linker added during cloning and is the first ATG located downstream from a stop codon. Amino acid sequences derived by Edman degradation of tryptic peptides purified from oat plasma membrane H^+-ATPase (19) are easily placed at various positions within the predicted *A. thaliana* sequence in pAHA1-11 (Fig. 2), confirming its identity as cDNA encoding the plasma membrane proton pump. In addition, amino acid sequence predicted for the 0.6-kb *Avena sativa* clone pTS.6 showed 89% identity to the *A. thaliana* sequence in the region from Trp-477 to Met-675.

Number of Plasma Membrane H^+-ATPase Genes in *A. thaliana*. In addition to the full-length cDNA clone pAHA1-11, a second clone (pAHA2) containing a 2.2-kb insert was obtained from the same *A. thaliana* library. Out of the 863 bp sequenced in pAHA2, there is 90% overall nucleotide identity with pAHA1-11, but, unlike pAHA1-11, pAHA2 is a partial clone lacking the coding sequence for approximately one-third of the amino-terminal end. The nucleotide mismatches between pAHA1-11 and pAHA2 are evenly distributed within the protein coding sequence but are more frequent in the 3′ untranslated regions.

A Southern blot analysis of *A. thaliana* genomic DNA was performed by using, as radioactive probe, a 930-bp *Pst* I fragment from the carboxyl-terminal half of the pAHA1-11 coding region. The enzymes chosen for these genomic digests cut the cDNA insert at least once but do not cut in the 930-bp fragment. The rationale for these digestions was that every hybridizing band observed would likely represent a separate gene, providing the 930-bp region was devoid of introns with the same restriction sites. As demonstrated in Fig. 3, both digestions gave two intense hybridizing bands, at 10 and 15 kb with *Ava* I plus *Stu* I (Fig. 3, lane 1) and at 4 and 10 kb with *Ava* I plus *Pvu* II in the other (Fig. 3, lane 2).

Structural Features Compared to Other 100,000-Da Cation ATPases. The protein sequence predicted from the nucleotide sequence of clone pAHA1-11 shows clear homology with 100,000-Da cation pumps isolated from bacterial, fungal, and animal species. Closest homology was found with the protein sequence of the plasma membrane H^+-ATPase in two fungi, *N. crassa* (9) and *S. cerevisiae* (10). In a comparison with either fungal protein, the plant protein showed 36% overall identity in amino acid sequence. Corresponding numbers for comparing pAHA1-11 with other cation pumps were 27% with the Ca^{2+}-ATPase of muscle sarcoplasmic reticulum (20), 24% with H^+,K^+-ATPase of stomach plasma membrane (21),

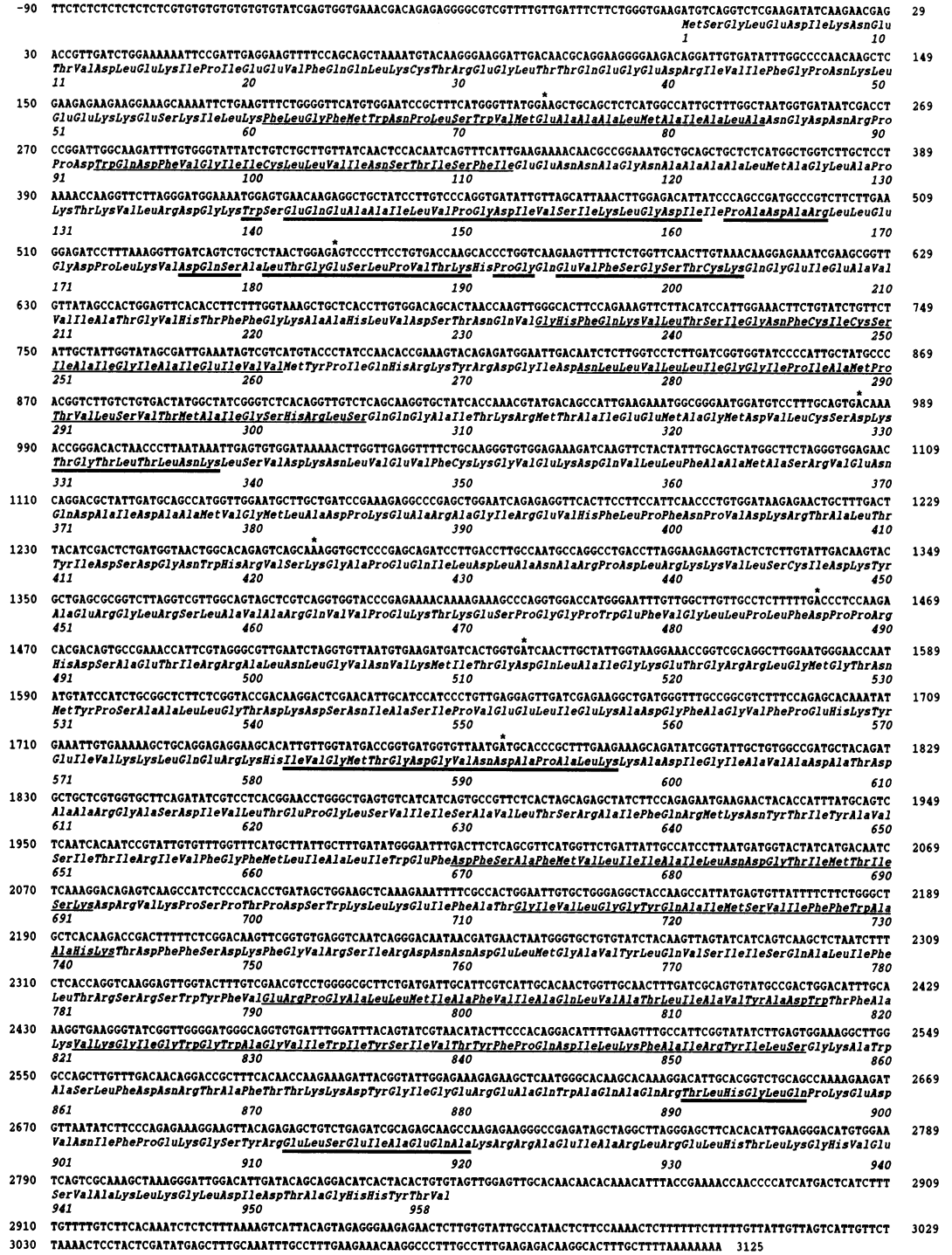

FIG. 2. Nucleotide sequence of the *A. thaliana* plasma membrane H^+-ATPase gene and the predicted amino acid sequence. The eight transmembrane segments suggested by a hydropathy plot are underlined. ★, Amino acid residues that are thought to play a role in catalytic activity, based on modification with covalent modifying reagents or by site-directed mutagenesis experiments with homologous 100,000-Da cation pumps (see text for explanation). Amino acids marked with a double underline are identical to residues observed in oat plasma membrane H^+-ATPase tryptic peptides sequenced by automated Edman degradations (19).

22% with the Na^+,K^+-ATPase of kidney plasma membrane (22), and 21% with the K^+-ATPase of bacterial plasma membrane (23).

The *A. thaliana* plasma membrane H^+-ATPase protein sequence was further analyzed by computing a hydropathy plot (24). As shown in Fig. 4, this analysis predicts the presence of eight transmembrane segments, a feature in common with the fungal and other 100,000-Da ATPases. In a side-by-side comparison of hydropathy plots with the *N. crassa* enzyme, the plant enzyme shows three differences. First, positioning of the plant sequence is displaced due to the absence of an amino-terminal segment *ca.* 60 residues long and the addition of a carboxyl-terminal segment of similar length. This displacement is confirmed by sequence homology matchups in conserved regions located between and within the transmembrane segments. Second, there is at least one, and possibly two, additional hydrophobic sequences adjacent to the fifth and sixth transmembrane segments in the plant enzyme. Third, the markedly deeper troughs corresponding to the first three predicted "extracellular loops" of

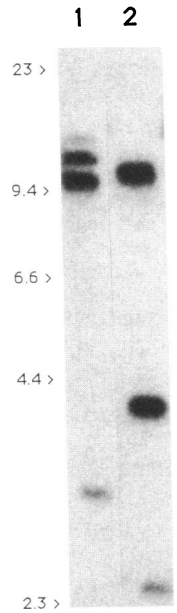

FIG. 3. Southern blot analysis of the *A. thaliana* plasma membrane H^+-ATPase. Genomic DNA digested with *Ava* I and *Stu* I (lane 1) or *Ava* I and *Pvu* II (lane 2) was electrophoresed and blotted. The hybridization probe was a gel-purified *Pst* I 930-bp fragment from pAHA1-11 labeled by nick-translation with [α-^{32}P]dCTP. The resulting autoradiogram is shown.

the plant enzyme show considerably greater hydrophilicity than that observed in the fungal enzyme. A visual examination of these sequences confirms that the increased hydrophilicity is caused by an increased frequency of charged residues.

To more precisely define conserved structures, the plant and fungal sequences were compared by using dot plot profiles (Fig. 5). This analysis demonstrates that the two fungal species *N. crassa* and *S. cerevisiae* have amino acid identity over their entire sequence, as shown by the nearly uninterrupted solid diagonal line. In contrast, the plant and

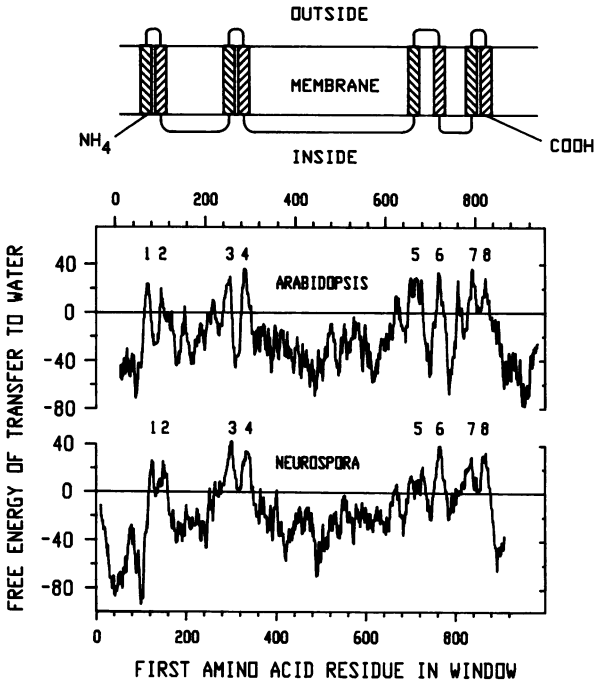

FIG. 4. Computer-assisted hydropathy plots of the *A. thaliana* and *N. crassa* (9) plasma membrane H^+-ATPase protein sequences. The plots were calculated according to Engelman *et al.* (24) with the PepPlot program (16). This program adds up the free energies of transfer of amino acids from water into oil over a running window of 20 residues. Values above the line represent hydrophobic segments, whereas those below the line are hydrophilic. A model for the predicted structure of the *A. thaliana* plasma membrane H^+-ATPase protein is shown above the plots.

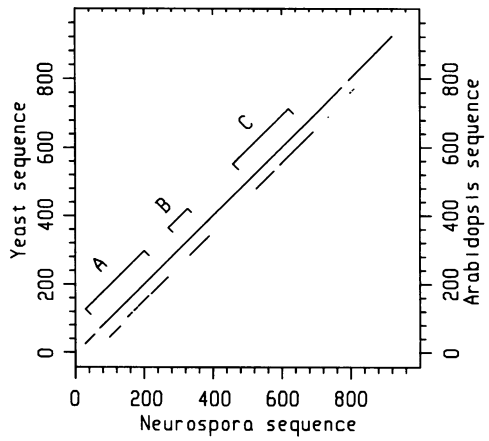

FIG. 5. Dot plot profiles comparing the plant and fungal plasma membrane proton pump amino acid sequences. This comparison used a symbol comparison table with perfect matches worth 1.5 points and mismatches assessed points based on the evolutionary closeness between the amino acids (25). The points were summed over a window of 30 amino acids, and if the total points were greater than 22.5, a dot was placed at the coordinates corresponding to the residue at the center of the window in each sequence. The two parallel diagonal lines are composed of dots arising from pairwise comparisons of either the two fungal enzymes, *N. crassa* (9) and yeast (10) (uninterrupted diagonal line), or the *Neurospora* enzyme compared to *Arabidopsis* (interrupted line below the diagonal). A, B, and C refer to conserved regions discussed in the text.

fungal enzymes display only three regions of high conservation: (*i*) region A, which includes transmembrane segments 1 and 2 plus the intervening 150-residue hydrophilic cytoplasmic loop that precedes transmembrane segment 3, (*ii*) region B, which includes transmembrane segment 4 and a 20-residue hydrophilic sequence that encompasses Asp-329, the site of phosphorylation during enzyme turnover, and (*iii*) region C, which includes the carboxyl-terminal half of the large 400-residue hydrophilic cytoplasmic loop that separates transmembrane segments 4 and 5.

DISCUSSION

The isolation of two cDNA clones encoding similar but distinct plasma membrane H^+-ATPase genes suggests that there are at least two separate genes encoding this enzyme in *A. thaliana*. The observation of multiple bands on Southern blot hybridizations is consistent with this interpretation. The high degree of nucleotide sequence conservation between the two cDNA clones suggests a recent evolutionary separation of the two genes.

Even though the H^+-ATPase is one of the more abundant proteins in the plasma membrane of higher plants, it constitutes only 0.01% of the total extractable cell protein (19). This concentration is 10–100 times lower than in fungal cell extracts and correlates with our observation of only 1–3 plant ATPase cDNA clones isolated per 100,000 recombinant cDNA clones screened. These calculations highlight the potential difficulty in isolating cDNA clones encoding other less abundant plant plasma membrane proteins.

The protein predicted from the nucleotide sequence of pAHA1-11 shows an overall similarity of 36% identity with the fungal plasma membrane H^+-ATPase. Three discrete regions contain greater sequence conservation. Chemical modification and genetic replacement studies have identified amino acids in these regions that may be essential for specific partial reactions within the catalytic cycle. For example, in the middle of the first transmembrane segment of region A is located Glu-74 of pAHA1-11, which is homologous to a dicyclohexylcarbodiimide-reactive amino acid also found in

the middle of the first transmembrane segment of the fungal enzyme and which may be involved in proton conduction (26). In the fungal gene, engineered replacement of a glutamic acid with glutamine in region A (Glu-184 of pAHA1-11) specifically reduces dephosphorylation, suggesting that region A also is involved in phosphatase activity (27). Region B contains Asp-329 of pAHA1-11, the site of phosphorylation of the reaction intermediate in the plant enzyme (28). Between regions B and C is located Lys-423 of pAHA1-11, which is homologous to a lysine in the Na^+,K^+-ATPase that reacts with fluorescein isothiocyanate in an ATP-protectable manner (29). Region C has been implicated by both chemical modification (30) and genetic studies (27) as the main site of nucleotide binding within this family of enzymes. Specifically, aspartyl residues at positions 487, 513, and 592 of pAHA1-11 are homologous to aspartyl residues in the yeast enzyme which, when mutated, cause changes in nucleotide specificity.

This study reveals three main differences between the *A. thaliana* and fungal plasma membrane proton pumps. First, the two enzymes show greatest divergence in amino acid sequence at the carboxyl termini. In fact, the plant enzyme shows an additional 60-residue carboxyl sequence that is missing in the fungal enzyme, as confirmed by both DNA sequence and Edman degradations of the purified plant enzyme. Second, the plant enzyme is missing a 60-residue amino-terminal sequence observed in the fungal enzyme. These changes offset each other and result in the two proteins showing only a small difference in overall length (959 versus 920).

A third difference between the plant and fungal enzymes is the higher frequency of charged residues in extracellular loops of the plant protein. The additional charged residues in the extracellular loops are composed of both acidic and basic residues. In consideration of their location facing the cell wall, it is possible that these charged amino acids are the site of functionally important interactions between the plant proton pump and the extracellular environment.

Hydropathy analysis of this plant enzyme is consistent with eight transmembrane segments, which suggests that the amino and carboxyl termini are both on the cytoplasmic side of the membrane. However, this analysis is inconclusive, particularly with respect to one or two additional hydrophobic stretches found in the plant enzyme's carboxyl terminus that are absent or less pronounced in the fungal protein. Confusion in delineating the exact number of transmembrane segments in the carboxyl terminus of the 100,000-Da cation-pumping ATPases further emphasizes the need to confirm this model with experimental data (5) as, for example, with antibodies directed to peptides predicted to be on the outside or inside membrane surfaces (31).

So far as we know, this represents the first gene for a plant plasma membrane protein to be cloned and sequenced, and its availability offers additional approaches in the study of plant plasma membrane function. Electrophysiological and biochemical studies suggest that the proton pump provides the driving force for nutrient transport across the plasma membrane. By mapping the H^+-ATPase genes by using restriction fragment length polymorphisms, it will be possible to test whether known genetic differences in mineral transport (32, 33) are linked to variant proton pumps. It is also possible that by using gene transformation techniques to modify the expression of this single enzyme, changes in the nutrient uptake capabilities of crop plants may be obtained. Finally, genetic experiments utilizing this cloned ATPase gene could provide a test of the hypothesis that ATPase-mediated changes in cytoplasmic or cell wall pH are a direct cause of hormone-stimulated growth in plants (34).

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