To examine the functional properties of the three major isoforms of plasma membrane H^+-ATPase expressed in Arabidopsis thaliana (AHA1, AHA2, and AHA3), we employed a system for the heterologous expression of functional plant plasma membrane H^+-ATPase in yeast (Villalba, J. M., Palmgren, M. G., Berberian, G. E., Ferguson, C., and Serrano, R. (1992) J. Biol. Chem. 267, 12341–12349). Each isoform was expressed efficiently but appeared to be retained in the endoplasmic reticulum of yeast. All isoforms displayed qualitatively similar enzymatic properties, but quantitative differences were found. When compared with AHA3, AHA1 and AHA2 had an apparent higher turnover rate for ATP hydrolysis, exhibited a 10-fold higher apparent affinity for ATP, and a 3-fold higher sensitivity toward vanadate. This study represents the first comparison of the functional properties of isoforms of the plant plasma membrane H^+-ATPase.

The plasma membrane H^+-ATPase is an integral membrane protein belonging to the family of P-type ATPases (Pedersen and Carafoli, 1987). It plays an essential role for establishing the electrochemical gradient of protons across the plasma membrane of plant and fungal cells. The ion gradient formed by this enzyme is necessary for the active transport of essential nutrients into cells (Serrano, 1989; Sussman and Harper, 1989). The plant plasma membrane H^+-ATPase is composed of a single subunit which exists as multiple isoforms. Isoforms of the plant plasma membrane H^+-ATPase are encoded for by multiple isoforms in many tissues. Concomitant expression of isoforms in the same cells or tissue is a deterrent in their characterization. To determine whether inherent functional differences exist among these isoforms, we have employed a recently developed system for heterologous expression of plant plasma membrane H^+-ATPase which permits the convenient analysis of enzymatic activity (Villalba et al., 1992). This system involves expression of functional plant H^+-ATPase in the endoplasmic reticulum of yeast cells. By employing this system, three A. thaliana H^+-ATPase isoforms have been produced individually in yeast in the same cellular environment. This has enabled us to analyze various biochemical parameters of the isoforms.

MATERIALS AND METHODS

Construction of Plasmids—Plasmids pRS-891, containing the AHA1 gene, and pMP-142, containing AHA2, have been described previously (Villalba et al., 1992; Palmgren and Christensen, 1993). pRS-541 was produced by cloning the AHA3 cDNA (Pardo and Serrano, 1991a) into pBluescript II. The AHA3 cDNA was a kind gift from J. M. Pardo and Professor R. Serrano (Universidad Politecnica, Valencia, Spain). A 1081-base pair EcoRI fragment of pRS-541, containing the ATG start codon and an internal BgIII site, was made blunt by Klenow treatment, and digested by BgIII. The 227-base pair digestion product which contained the initiation ATG was subcloned simultaneously with the 5.0-kb BgIII-KpnI fragment of pRS-541, containing the rest of the AHA3 gene.

Functional Comparisons between Plant Plasma Membrane H^+-ATPase Isoforms Expressed in Yeast*

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J. Harper, personal communication.

The abbreviations used are: ER, endoplasmic reticulum; lyso-PC, lysophosphatidylcholine; MES, 4-morpholinoethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
into EcoRV-KpnI-digested pBluescript SK- (Stratagene) to produce pMP-137. These steps removed eight spurious out-of-frame ATG codons 5' to the correct ATG. The complete AHA3 gene was excised as a 3.2-kb EcoRI fragment, subcloned into pBluescript SK-, digested partially with EcoRI, treated with Klenow, and excised as a 3.2-kb EcoRI-SpeI fragment by SpeI. This fragment was ligated to the 7.6-kb (XhoI)-SpeI fragment of pRS-136, in which the XhoI sticky end had been blunted by Klenow treatment, in order to produce pMP-169.

**Yeast Strains and Culture Conditions**—The yeast strains used in this study were: RS-72 ([Cid et al., 1987], RS-933 (RS-72/YPep351 (Hill et al., 1986)), RS-904 (RS-72/pRS-891 (Villalba et al., 1992)), MP-142 (RS-72/pMP-142 (Palmgren and Christensen, 1993)), and MP-170 (RS-72/pMP-169). Yeast cells were made competent for plasmid uptake by treatment with lithium acetate and polyethylene glycol according to Ito et al. (1983). The synthetic growth media were as described (Villalba et al., 1992). In order to express only plasmid-born enzyme, yeast cells were cultured in galactose-containing medium until growth reached the stationary phase after which they were stored at 4°C without shaking. After incubation in the cold for 24 h, the cells were pelleted, resuspended in the same volume of glucose medium (30°C), and cells were harvested after shaking at 30°C for 1 h. Upon prolonged growth in glucose medium, the level of AHA3 expressed diminished significantly (to below 25% of maximum; Fig. 1), while expression of AHA1 and AHA2 remained the same (data not shown). The reason for this instability of AHA3 is unknown, and no proteolytic degradation of the polypeptide could be observed in Western blots immunodecorated with a monoclonal plant H+-ATPase antibody (Villalba et al., 1991; Fig. 1) or with polyclonal antisera directed against the N terminus, the central part, and the C terminus of AHA3 (Palmgren et al., 1991; data not shown).

**Biochemical Methods**—For analysis of subcellular distribution of ATPase, a microsomal membrane fraction prepared by differential centrifugation according to Villalba et al. (1992) was layered on top of a continuous gradient composed of 20–53% (w/v) sucrose in 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 1 mM dithiothreitol. After overnight centrifugation at 30,000 revolutions/min (Beckman rotor TI-SW40), fractions of 1 ml were collected from the top of the gradient.

For purification of the ER fraction containing plant ATPase, a microsomal membrane fraction from 250 ml of glucose culture (see above) was applied to a discontinuous sucrose gradient made of 5 ml of 29% (w/w) sucrose and 5 ml of 34% (w/w) sucrose. After overnight centrifugation at 30,000 revolutions/min (Beckman rotor TI-SW40), membranes enriched in plant plasma membrane H+-ATPase were recovered at the 29/34 interface.

ATP hydrolysis was measured at 30°C in 0.3 ml of reaction mixture containing 50 mM MOPS adjusted to pH 6.5 with Tris, 3 mM MgCl₂, 0.5 mM NaN₃, 50 mM KNO₃, 0.1 mM EDTA, 5 mM sodium azide (to inhibit mitochondrial ATPase), 0.2 mM ammonium heptamolybdate (to inhibit acid phosphatase). Stocks of vanadate were boiled for 10–30 min as described (Baginski et al., 1967). SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described (Villalba et al., 1992). A monoclonal antibody against plant plasma membrane H+-ATPase was utilized. Protein was measured according to Bradford (1976).

**RESULTS**

**Expression of Plant H+-ATPase Isoforms under Different Conditions**

Our objective in this study was to determine the enzymatic properties of three isoforms of the plasma membrane H+-ATPase expressed in the plant A. thaliana. Expression of plant plasma membrane H+-ATPase in yeast cells has proven to be a very powerful system for addressing the relationship between structure and function for this enzyme (Villalba et al., 1992; Palmgren and Christensen, 1993). This system allows the characterization of heterologously expressed plant H+-ATPase in membranes devoid of endogenous yeast ATPases (Villalba et al., 1992; Palmgren and Christensen, 1993). The A. thaliana isoform genes were cloned in multicopy expression plasmids under control of the promoter of the yeast plasma membrane H+-ATPase gene (PMA1). This promoter confers high levels of constitutive expression and is, in addition, positively regulated by glucose (Eraso et al., 1987; Capieux et al., 1988). The expression plasmids are introduced into Saccharomyces cerevisiae strain RS-72 ([Cid et al., 1987], 1993). In this strain the constitutive promoter of the chromosomal PMA1 gene has been placed under control of a galactose-dependent promoter by a gene disruption strategy (Cid et al., 1987). The resulting transformed strains (strain RS-934 expressing AHA1 (Villalba et al., 1992), strain MP-142 expressing AHA2 (Palmgren and Christensen, 1993), and strain MP-170 expressing AHA3 (this paper)) would express yeast ATPase on galactose medium but not on glucose medium. The plant ATPases would be expressed on both media, but at somewhat higher levels on glucose medium. In addition, yeast cells had been transformed with the plasmid lacking the plant cDNA (control strain RS-933) or with plasmid containing the yeast H+-ATPase gene (control strain MP-213).

In order to investigate the expression of plant H+-ATPase isoforms, yeast cells putatively expressing AHA1, AHA2, AHA3, as well as the control strain RS-933 were cultured in galactose medium until growth reached the stationary phase. Total membranes from all four strains were analyzed by Western blot (Fig. 1). All three plant plasma membrane H+-ATPase isoforms were expressed at high levels in the yeast cells. The AHA2 isoform migrated with slightly greater mobility than isoforms AHA1 and AHA3 on 7% acrylamide-SDS gels (Fig. 1). This is surprising since the predicted molecular masses of the ATPase isoforms from A. thaliana (Pardo and Serrano, 1989b) all are about 104 kDa: AHA1 = 104,182; AHA2 = 104,270; AHA3 = 104,318). Other features than size that could influence mobility in SDS-polyacrylamide gel electrophoresis could be amount of SDS binding, structural features retained in SDS, or post-translational modifications. In order to rule out trivial explanations, perhaps caused by cloning artifacts, we sequenced the coding region of the AHA2 clone from both ends and the terminal sequences corresponded to the published sequences (Pardo and Serrano, 1989b; Harper et al., 1990). At the protein level, attempts to sequence the purified AHA2 protein by Edman degradation were unsuccessful since the N terminus appeared to be blocked.

**Growth Phenotype of Yeast Cells Expressing Plant H+-ATPase Isoforms**

In order to repress the expression of endogenous yeast ATPase and boost that of plant ATPase, cells were transferred...
to glucose medium. It is expected that expression of functional plant H+-ATPase in the yeast plasma membrane should support growth of cells devoid of yeast ATPase. However, among the three *A. thaliana* H+-ATPase isoforms only one, AHA2, was able to complement endogenous yeast plasma membrane H+-ATPase (PMA1), albeit to a very low degree (Fig. 2), as reported previously (Palmgren and Christensen, 1993).

The lack of complementation exhibited by AHA3 suggested that this particular isoform is retained in the ER of transformed yeast cells as is the case for the AHA1 (Villaalba et al., 1992) and AHA2 polypeptides (Palmgren and Christensen, 1993).

**Different Distribution of Yeast H+-ATPase and AHA3**

Total membranes from yeast cells grown on glucose for 16 h were fractionated by sucrose gradient centrifugation under isopycnic conditions. Membranes from strain MP-170 expressing AHA3 contained a large peak of plasma membrane H+-ATPase activity at 30% sucrose with a shoulder at 40–45% sucrose (Fig. 3). This peak correspond to a novel fraction of enlarged ER (Palmgren and Christensen, 1993). The low level of the AHA3 expressed in the ER as compared with AHA1 and AHA3 might explain why only this particular isoform complements yeast cells devoid of yeast ATPase (Fig. 2). Assuming that the ER of yeast has a maximal capacity for retaining polypeptides, the ER harboring this specific isoform may be saturated with plant H+-ATPase thus allowing for a certain nonspecific "spill-over" of enzyme to the plasma membrane (Palmgren and Christensen, 1993).

**Characterization of the Plant Plasma Membrane H+-ATPase Isoforms Synthesized in Yeast**

**Specific Activity**—Each of the AHA species expressed in the ER of transformed yeast cells were active ATPases as illustrated in Table 1. The specific activity of each isoform in ER vesicles purified from yeast cells transferred to glucose medium for 1 h only and assayed at pH 6.5 and 3 nm ATP varied significantly, and the specific activity of AHA2 was about 2-fold higher than that of AHA1 and AHA3 (Table 1).

**Expression of Plant Plasma Membrane H+-ATPase Isoforms in the ER of Yeast**

Analysis of the polypeptide composition of membrane vesicles derived from the endoplasmic reticulum of yeast cells transformed with each of the different *A. thaliana* plasma membrane H+-ATPase constructs used in this paper revealed that each isoform was expressed at high levels in this particular membrane fraction (Fig. 4A). Although the expression level varied somehow from membrane preparation to membrane preparation, AHA2 was always expressed to the highest degree followed by AHA3 and AHA1. Densitometric analysis indicated that the 95 kDa AHA2 band amounted to about 70% of total ER protein, while AHA3 and AHA1 were expressed to somewhat lower degrees (about 55 and 40% of total ER protein, respectively). Interestingly, as for total membranes (see above), in our gel system AHA2 migrated with an apparent size smaller than predicted from its sequence compared with the other isoforms.

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**Different Distribution**

FIG. 2. Drop test for the growth of yeast strains expressing plant plasma membrane H+-ATPase isoforms. A, control strain MP-213 (expressing yeast ATPase on both galactose and glucose media); B, control strain RS-933 (expressing yeast ATPase only on galactose medium); C, strain RS-934 (expressing AHA1 on both media and yeast ATPase only on galactose medium); D, strain MP-142 (expressing AHA2 on both media and yeast ATPase only on galactose medium); E, strain MP-170 (expressing AHA3 on both media and yeast ATPase only on galactose medium). Cells were grown to saturation on galactose medium, and about 103 cells in 10 μl were spotted on agar plates containing galactose or glucose media as indicated. Growth was recorded after 4 days.

**Distribution of H+-ATPase activity in sucrose gradient fractions.** Total membranes were isolated from yeast grown on glucose medium for 16 h expressing either *A. thaliana* H+-ATPase isoform AHA3 (strain MP-170), plasmid-borne yeast H+-ATPase (C, strain MP-215), or no plasma membrane H+-ATPases (A, strain RS-933) and loaded on a linear sucrose gradient. After overnight centrifugation, 1-ml fractions were collected from the top of the gradient and used for ATPase analysis.
FIG. 4. Polypeptide composition of ER membrane vesicles from transformed yeast cells expressing plant plasma membrane H+-ATPase isoforms. A, purified membranes (10 μg) from control strain RS-933 (lane 1), expressing no plasma membrane H+-ATPases, strain RS-934 (lane 2, expressing AHA1), strain MP-142 (lane 3, expressing AHA2), and strain MP-170 (lane 4, expressing AHA3) grown in glucose media for 1 h were run on SDS-polyacrylamide gels and stained with Coomassie Blue. The ATPase activity of each membrane preparation (in P/mg protein/min) was C, 0.00 unit; 1, 0.70 unit; 2, 2.95 units; 3, 0.57 units. B, equal amounts of ATPase activity (10 μl of ER membrane preparation; 0.2 μmol of P; released/min/ml) from strain RS-934 (lane 1, expressing AHA1), strain MP-142 (lane 2, expressing AHA2), and strain MP-170 (lane 3, expressing AHA3) were run on SDS-polyacrylamide gels and stained with Coomassie Blue. The position of heterologously expressed plant H+-ATPases is indicated by arrows. In the lower panel the various plant H+-ATPase polypeptides present in the membranes were visualized by a densitometric scan of the gels shown above. Relative abundancies (in percent of AHA1 polypeptide) are indicated.

The cloning of the cDNAs for the three major Arabidopsis plasma membrane H+-ATPase isoforms (Harper et al., 1989; Pardo and Serrano, 1989a, 1989b) has allowed for their individual expression in the ER of yeast, thereby producing a means in which to characterize the kinetic properties of each isoform. Studying the properties of the three plant ATPase isoforms expressed in yeast is advantageous in that they are all present in the same environment. That is, lipid composition of the surrounding membrane is similar. Also, if cell type-specific
Functional Comparison of Plasma Membrane $H^+$-ATPase Isoforms

### Table I

Specific activities of *Arabidopsis* $H^+$-ATPase isoforms expressed in yeast ER

ATPase activities were assayed at $30^\circ C$ in 300 ml of reaction mixture containing 50 mM MOPS-BTP, pH 6.5, 3 mM Na$_2$ATP, 10 mM MgCl$_2$, 50 mM KNO$_3$, 5 mM sodium azide, 0.2 mM ammoniumheptamolybdate, and 0.1 mM EDTA. ER membrane vesicles were isolated from yeast cells grown for 1 h on glucose medium in order to boost expression of plant ATPase. The data shown are average values for seven independent ER membrane preparations.

| Strain or Isoform | RS-933 | RS-934 | MP-142 | MP-170 |
|-------------------|--------|--------|--------|--------|
| $\mu$mol Pi/min/mg | $0.1 \pm 0.1$ | $1.2 \pm 0.5$ | $2.4 \pm 0.6$ | $1.1 \pm 0.5$ |

### Table II

Effect of Mg$^2+$ and Ca$^{2+}$ on plant $H^+$-ATPase expressed in yeast ER

Activities were assayed at $30^\circ C$ in the absence or presence of the indicated concentrations of MgCl$_2$ and CaCl$_2$ in 300 ml of reaction mixture containing 50 mM MOPS-BTP, pH 6.5, 3 mM Na$_2$ATP, 50 mM KNO$_3$, 5 mM sodium azide, 0.2 mM ammonium heptamolybdate, and 0.1 mM EDTA. The data shown are the average of three determinations obtained from one ER membrane isolation.

| Isoform | AHA1 Activity | AHA2 Activity | AHA3 Activity |
|---------|---------------|---------------|---------------|
| MgCl$_2$ | $1.49 \pm 0.02$ | $2.10 \pm 0.01$ | $1.12 \pm 0.03$ |
| CaCl$_2$ | $0.90 \pm 0.01$ | $0.00 \pm 0.01$ | $0.00 \pm 0.01$ |
| MgCl$_2$ + CaCl$_2$ | $1.33 \pm 0.11$ | $1.73 \pm 0.09$ | $1.00 \pm 0.06$ |
| No additions | $0.78 \pm 0.03$ | $1.23 \pm 0.04$ | $0.80 \pm 0.05$ |

Fig. 5. H$^+$ dependence of $H^+$-ATPase activity. Data are expressed as percent of total $H^+$-ATPase activity. The experiment is typical among seven experiments involving separate membrane preparations. The $H^+$-ATPase activities at pH 6.5 (100%) for each preparation used in this experiment were as follows (units are micromoles of Pi/mg protein/min): AHA1 = 0.88; AHA2 = 2.46; AHA3 = 0.84. Symbols are as follows: $\bullet$, AHA1; $\circ$, AHA2; $\square$, AHA3.

Modification of isoforms differ either at the RNA level (i.e., variable splicing) or at the protein level (i.e., regulatory phosphorylation), this may be minimized when all three isoforms are expressed in a heterologous species such as yeast.

Electrophoresis of ER isolated from transformed yeast cells clearly reveal that the known Arabidopsis plasma membrane $H^+$-ATPase isoforms migrate with slightly different rates through the acrylamide-SDS gel system (Figs. 1 and 4). Biochemical heterogeneity in plant plasma membrane $H^+$-ATPase preparations was first reported for native corn root plasma membrane $H^+$-ATPase (Gallagher and Leonard, 1987). Two closely associated ATPase bands, both reacting with antibodies to Neurospora or plant plasma membrane $H^+$-ATPase, have also been demonstrated in the plasma membrane isolated from barley roots (Dupont et al., 1988), corn roots (Grouzis et al., 1990), oat roots (Palmgren et al., 1990), sugar beet leaves, and A. thaliana (Palmgren et al., 1991). Anomalous migration in SDS-polyacrylamide gel electrophoresis is a feature of other P-type ATPases. The $\alpha$-subunit of the Na$^+$/K$^+$-ATPase (Sweedner 1989, 1990) and the SERCA1 isoform of sarcoplasmic reticulum Ca$^{2+}$-ATPase (Lytton et al., 1992) both migrate with an apparent size larger than predicted from their sequence compared with isoforms of the same enzymes having only slightly different calculated molecular weights. Na$^+$/K$^+$-ATPase $\alpha$-subunit mobility seems to reflect complex detergent-protein interactions that can be affected by experimental conditions and the existence of more than one band on gels may reflect different conformations of each isoform in detergent (Sweedner, 1990). Therefore, in light of the results presented here, what is seen in plant plasma membrane preparations as $H^+$-ATPase doublets seems to be a direct visualization of the isoform heterogeneity of the preparation.

Why isoforms of the plasma membrane $H^+$-ATPase? At least AHA2 (Sussman, 1992) and AHA3 (DeWitt et al., 1991) are expressed in a tissue- and developmental specific manner. Lingrel (1992) has pointed to two models of Na$^+$/K$^+$-ATPase $\alpha$
The plant plasma membrane $H^+$-ATPase is a phosphoprotein in vivo, and it has been hypothesized that kinase-mediated phosphorylation of the ATPase is the means by which the catalytic activity is altered post-translationally (Schaller and Susman 1988). In the presence of Mg$^{2+}$, the $H^+$-ATPase activity of oat root plasma membrane vesicles is inhibited by Ca$^{2+}$ (80% inhibition at 1 mM; Vara and Serrano, 1982). When purified oat root plasma membrane vesicles were incubated with [$\gamma$-32P]ATP, radioactivity was incorporated into the ATPase, at serine and threonine residues. The majority of this kinase-mediated ATPase phosphorylation was found to be strictly dependent on the presence of calcium (Schaller and Susman 1988). Taken together, these data suggest that the negative effect of Ca$^{2+}$ on the $H^+$-ATPase activity, if any, is indirect and sustained by a phosphorylation mechanism. Accordingly, none of the A. thaliana plasma membrane $H^+$-ATPase isoforms produced in yeast were sensitive to Ca$^{2+}$ (Table II).

The plant plasma membrane $H^+$-ATPase belongs to a super family of cation-translocating ATPases, the P-type ATPases (Pedersen and Carafoli, 1987). The properties of isoforms of other P-type ATPases have been compared by means of heterologous expression systems. The isoforms that have been characterized are the $\alpha_1$, $\alpha_2$, and $\alpha_3$-isoforms of Na$^+/K^+$-ATPase (Jewell and Lingrel, 1993; Bianco et al., 1993) and the SERCA1, SERCA2a, SERCA2b, and SERCA3 isoforms of sarcoplasmic reticulum Ca$^{2+}$-ATPase (Lytton et al., 1992; Verboom et al., 1992). The isoforms of each ATPase display qualitatively similar enzymatic properties but the quantitative properties of Na$^+/K^+$-ATPase and Ca$^{2+}$-ATPase isoforms are not identical in all respects. Isoforms of Na$^+/K^+$-ATPase and Ca$^{2+}$-ATPase, respectively, exhibit significant differences in affinities for their various ligands, thus supporting the hypothesis that the purpose of multiple isoforms is to provide ATPases with unique catalytic or regulatory properties in the various differentiated cells in which each is specifically expressed.

This study represents the first comparison of the functional properties of plant plasma membrane $H^+$-ATPase isoforms. If the isoforms in Arabidopsis possess the substrate dependence properties we have observed in yeast, it is tempting to speculate about their physiological significance. AHA3 is expressed in phloem cells and has an unusually low affinity for ATP, suggesting that these cells maintain an elevated ATP which...
would allow this particular isofrom to operate at a significant rate.

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