

# The Two Major Types of Plant Plasma Membrane H<sup>+</sup>-ATPases Show Different Enzymatic Properties and Confer Differential pH Sensitivity of Yeast Growth<sup>1</sup>

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The proton-pumping ATPase (H<sup>+</sup>-ATPase) of the plant plasma membrane is encoded by two major gene subfamilies. To characterize individual H<sup>+</sup>-ATPases, PMA2, an H<sup>+</sup>-ATPase isoform of tobacco (*Nicotiana plumbaginifolia*), was expressed in *Saccharomyces cerevisiae* and found to functionally replace the yeast H<sup>+</sup>-ATPase if the external pH was kept above 5.0 (A. de Kerchove d'Exaerde, P. Supply, J.P. Dufour, P. Bogaerts, D. Thinès, A. Goffeau, M. Boutry [1995] J Biol Chem 270: 23828–23837). In the present study we replaced the yeast H<sup>+</sup>-ATPase with PMA4, an H<sup>+</sup>-ATPase isoform from the second subfamily. Yeast expressing PMA4 grew at a pH as low as 4.0. This was correlated with a higher acidification of the external medium and an approximately 50% increase of ATPase activity compared with PMA2. Although both PMA2 and PMA4 had a similar pH optimum (6.6–6.8), the profile was different on the alkaline side. At pH 7.2 PMA2 kept more than 80% of the maximal activity, whereas that of PMA4 decreased to less than 40%. Both enzymes were stimulated up to 3-fold by 100 µg/mL lysophosphatidylcholine, but this stimulation vanished at a higher concentration in PMA4. These data demonstrate functional differences between two plant H<sup>+</sup>-ATPases expressed in the same heterologous host. Characterization of two PMA4 mutants selected to allow yeast growth at pH 3.0 revealed that mutations within the carboxy-terminal region of PMA4 could still improve the enzyme, resulting in better growth of yeast cells.

The plasma membrane H<sup>+</sup>-ATPase plays a pivotal role in plant physiology. By extruding protons from the cell, an electrochemical gradient is created across the plasma membrane, providing the driving force for ion and nutrient uptake, intracellular pH regulation, maintenance of cell turgidity, and related phenomena such as cell growth, organ and stomata movement, and salinity tolerance (for review, see Serrano, 1989; Sussman, 1994; Michelet and Boutry, 1995; Palmgren, 1998).

The plant H<sup>+</sup>-ATPase is encoded by a multigene family of approximately 10 genes (Harper et al., 1990, 1994; Perez et al., 1992; Ewing and Bennett, 1994). However, all of the

H<sup>+</sup>-ATPase genes that have been identified at the cDNA level in several species can be classified into two subfamilies according to their sequence identity. Divergence between these subfamilies that represent the most highly expressed genes seems to predate the evolution of current monocotyledonous and dicotyledonous species (Moriau et al., 1993).

Biochemical characterization of individual H<sup>+</sup>-ATPase isoforms and the unraveling of their possible differences in structure, kinetics, and regulatory features will help us understand the respective roles of the two major H<sup>+</sup>-ATPase subfamilies. Unfortunately, this kind of study is hindered in plants because of the simultaneous presence of several isoforms within a single organ. However, the heterologous expression of individual H<sup>+</sup>-ATPases in the yeast *Saccharomyces cerevisiae* has provided a way of overcoming this difficulty. Three Arabidopsis H<sup>+</sup>-ATPase genes, *AHA1*, *AHA2*, and *AHA3*, all belonging to the second subfamily, have been expressed in yeast (Villalba et al., 1992; Palmgren and Christensen, 1994). *AHA1* and *AHA3* did not allow growth and *AHA2* permitted slow growth when the yeast H<sup>+</sup>-ATPase gene, under the control of a Gal-induced promoter, was turned off. Characterization of the three gene products that accumulated in ER-derived internal membranes revealed quantitative differences in enzymatic properties (Palmgren and Christensen, 1994).

On the other hand, *pma2*, a *Nicotiana plumbaginifolia* H<sup>+</sup>-ATPase gene from the first subfamily, complemented a strain of *S. cerevisiae* deprived of its H<sup>+</sup>-ATPase genes if the external pH was kept above 5.0 (de Kerchove d'Exaerde et al., 1995). Therefore, this expression system made it possible to characterize biochemically a plant H<sup>+</sup>-ATPase in a purified plasma membrane fraction devoid of contaminant yeast H<sup>+</sup>-ATPase. It was thus important to test a *N. plumbaginifolia* isoform of the second subfamily in the same expression system.

In the present study we show that the plant *pma4* also successfully replaced the yeast H<sup>+</sup>-ATPase gene and supported yeast growth. However, in contrast to the strain expressing *pma2*, which is unable to grow at an external pH lower than 5.0, the yeast strain containing *pma4* can grow at a pH as low as 4.0. Biochemical characterization of PMA4 and PMA2 revealed differences in enzymatic properties,

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Abbreviation: LPC, lysophosphatidylcholine.

pH sensitivity, and LPC stimulation. In addition, we selected two *pma4* mutants with improved ATPase, which allow yeast growth at an external pH of 3.0.

## MATERIALS AND METHODS

### Chemicals

5-Fluoroorotic acid was purchased from PCR, Inc. (Gainesville, FL), Na<sub>2</sub>ATP from Sigma, protease inhibitors from Boehringer Mannheim, yeast extract from KAT (Ohly, Hamburg, Germany), and yeast nitrogen base amino acids from Difco (Detroit, MI). All other reagents were of analytical grade.

### Plasmid Construction

The *pma4* cDNA from *Nicotiana plumbaginifolia*, *cpma4* described by Moriau et al. (1993), was cloned as a 3363-bp *EcoRI* restriction fragment in the plasmid vector PTZ19U starting 105 bp upstream from the initiation ATG of the PMA open reading frame and extending 353 bp beyond the stop codon. Within the 5' untranslated region is a small, 5-codon upstream open reading frame located 83 nucleotides upstream from the start of the PMA open reading frame. To prevent any potential interference of the 5' and 3' untranslated regions in the expression of the *pma4* gene in yeast, these were eliminated by PCR.

In the 5' region we made use of a *Bam*HI restriction site in the plasmid and of a *Pst*I site 371 nucleotides downstream of the PMA4 initiation codon. Two oligonucleotides, one corresponding to nucleotides -14 to +5 of the PMA4 translation initiation codon (CGCGGATCCGTG-GAGCAGAGATGGC; the introduced *Bam*HI site is underlined), and the other complementary to nucleotides 445 to 427 downstream of the PMA4 translation initiation codon (CGGCTTCCTGTTCACTCC), were synthesized to amplify the corresponding region in between. This amplified fragment, digested with *Bam*HI and *Pst*I, was then used to replace the corresponding original *Bam*HI-*Pst*I fragment, producing a new 5' region without the upstream open reading frame.

The same strategy was applied for the removal of the 3' untranslated region using a *Hind*III site in the plasmid and an *Afl*III site 417 nucleotides upstream of the stop codon. Two oligonucleotides, one corresponding to a sequence upstream of the *Afl*III site (-369 to -351 upstream of the stop codon; AGCATTATTTTCTACCTC) and the other complementary to nucleotides +18 to +36 downstream of the stop codon (CGCAAGCTTCTCCTTCTTGTGT; the introduced *Hind*III site is underlined), were synthesized for PCR amplification. The amplified fragment was then introduced to replace the original *Afl*III-*Hind*III region. The correct sequence of the modified *pma4* cDNA was verified by sequencing.

This modified *pma4* was then released as a *Bam*HI-*Hind*III fragment and introduced into the yeast Yeplac181 plasmid (bearing the 2 $\mu$  origin of replication and the *LEU2* marker) containing the promoter region of the yeast *PMA1* gene (de Kerchove d'Exaerde et al., 1995). The

transcription-terminator region of the yeast *PMA1* gene was then released as a 1.2-kb *Xba*I fragment, blunted by Klenow DNA polymerase, and inserted into the *Hind*III site (also blunted by the Klenow polymerase) of Yeplac181, yielding 2 $\mu$ p(*PMA1*)*pma4*.

### Culture Conditions

Yeast was grown at 30°C in a rich medium containing 2% (w/v) yeast extract and 2% Glu (YGlu medium) or Gal (YGal medium), or in a synthetic medium containing 0.7% yeast nitrogen base without amino acids, 0.11% drop mix (Trecos, 1989), all of the amino acids except those used for selection (His, Leu, Ura, and Trp), and 2% Glu (MGLu-His,Leu,Ura,Trp) or 2% Gal (MGal-His,Leu,Ura,Trp). Solid media also contained 2% agar (Difco). These media were supplemented with 20 mM K<sub>2</sub>HPO<sub>4</sub> and buffered to the pH indicated using HCl or KOH.

### Yeast Strains

Several yeast strains were used in this study. YPS14-4 (Supply et al., 1993) was the wild-type *S. cerevisiae*. YAK2 (de Kerchove d'Exaerde et al., 1995) was deleted from its own H<sup>+</sup>-ATPase genes, *PMA1* and *PMA2*, and survived with the yeast *PMA1* under the control of the *GAL1* promoter in a centromeric plasmid PRS-316 (Sikorski and Hieter, 1989). YAKpma2 (de Kerchove d'Exaerde et al., 1995) corresponds to YAK2, in which the centromeric plasmid bearing the yeast *PMA1* was replaced by a multi-copy plasmid bearing the plant *pma2* cDNA under the control of the yeast *PMA1* promoter. The strain YAKpma4 was obtained as follows: YAK2 was transformed with 2 $\mu$ p(*PMA1*)*pma4* (see above) and was plated on MGal-His,Leu,Trp,Ura. Transformants were replicated on MGLu-His,Leu,Trp medium containing 0.1% 5-fluoroorotic acid to delete the strain of the *URA3* plasmid pRS-316 containing the yeast *PMA1* gene under the control of the *GAL1* promoter. Loss of this plasmid was verified by Southern-blot analysis. Yeast cells were transformed after treatment with lithium acetate and PEG according to the method of Ito et al. (1983).

### Selection of Mutants

Several single colonies of the YAKpma4 strain were inoculated individually into 5 mL of YGlu medium, pH 5.5, and grown until they reached the stationary phase. After centrifugation and resuspension in 200  $\mu$ L of water, the yeast cells from each culture were plated onto solid YGlu medium, pH 3.0. Spontaneous mutants growing under these nonpermissive conditions appeared after 3 d at 30°C.

### Sequencing of *pma4* and Its Mutants

The 2 $\mu$ p(*PMA1*)*pma4* plasmid from both wild-type and mutant yeast strains was retrieved and transferred to *Escherichia coli*. The plasmid DNA was sequenced using a series of synthetic primers scattered throughout the *pma4* gene. The wild-type and mutated 2 $\mu$ p(*PMA1*)*pma4* were finally

reintroduced into the YAK2 strain to verify the phenotype they conferred.

### Plasma Membrane Preparation and Protein Determination

Plasma membranes were prepared according to the method of Goffeau and Dufour (1988). Yeast cells were grown in a 1.5-L culture (YGlu medium) and harvested at a density of  $80 \times 10^6$  to  $100 \times 10^6$  cells/mL. The cell pellet was then washed three times with ice-cold water and resuspended (1.5 mL/g fresh weight) in 250 mM sorbitol, 1 mM MgCl<sub>2</sub>, 50 mM imidazole, pH 7.5 (NaOH), 1 mM PMSF, and 5 mM DTT. After disruption, subcellular fractionation, and plasma membrane enrichment, the plasma membranes were finally resuspended in 10 mM imidazole, pH 7.5 (NaOH), 1 mM MgCl<sub>2</sub>, and 1 mM PMSF, and then divided into aliquots, frozen in liquid nitrogen, and stored at -80°C. The protein concentration was determined using the Bradford method (1976) with BSA as the standard.

### ATPase Assays

ATPase activity was assayed at 30°C in 50  $\mu$ L of a medium containing 6 mM MgATP, 1 mM free Mg<sup>2+</sup> (MgCl<sub>2</sub>), 50 mM Mes-NaOH, pH 7.0, 10 mM sodium azide (a mitochondrial ATPase inhibitor), 0.2 mM Na molybdate (a phosphatase inhibitor), 20 mM KNO<sub>3</sub> (a vacuolar ATPase inhibitor), and 4  $\mu$ g of proteins. The reaction was stopped after 12 min by the addition of 60  $\mu$ L of 5% TCA, 30  $\mu$ L of 50% NH<sub>4</sub> molybdate (in 4 N H<sub>2</sub>SO<sub>4</sub>), 30  $\mu$ L of 1% aminonaphthosulfonic acid, and 3% NaHSO<sub>3</sub>. A<sub>700</sub> was read after 15 min.  $K_m$  and  $V_{max}$  were determined using an ATP-regenerating system (100  $\mu$ g/mL pyruvate kinase and 5 mM PEP). The concentration of MgATP varied between 40  $\mu$ M and 10 mM.  $K_m$  and  $V_{max}$  were determined using Eadie-Hofstee plots. The optimal pH was determined as described above except that three buffers were mixed (50 mM Mes, 50 mM Mops, and 50 mM Tris) and adjusted to the pH indicated with HCl or NaOH. The amounts of ATP and Mg<sup>2+</sup> to be added to the reaction mixture at the pH indicated were calculated as described by Wach et al. (1990) to obtain the desired concentration of MgATP and Mg<sup>2+</sup>. All data presented in the figures represent the means  $\pm$  SD of at least three independent experiments using two separate membrane preparations.

### Measurement of Glu-Induced Acid Efflux from Yeast Cells

Yeast cells were grown in a 100-mL culture (YGlu medium at pH 5.5 [YAKpma4] or 6.5 [YAKpma2]), harvested at the late-exponential phase, washed four times with ice-cold water, and stored on ice. For each assay 10<sup>9</sup> cells were incubated in 10 mL of 250 mM sorbitol at 30°C in a vial with a magnetic barrel and a pH electrode of a microprocessor pH meter (Wissenschaftlich-Technische Werkstätten, Weiheim, Germany). Once a stable pH baseline was established (after about 5 min), 250 mM Glu was added as an energy source and the external pH was recorded over time.

### SDS-PAGE

Proteins (30  $\mu$ g) were suspended in a buffer containing 60 mM Tris-HCl, pH 6.8, 5% (w/v) glycerol, 1% (w/v) SDS, 10 mM DTT, 1 mM PMSF, 2  $\mu$ g/mL each of leupeptin, aprotinin, antipain, pepstatin, and chymotrypsin, and 0.005% bromphenol blue. The samples were kept on ice for 15 min and centrifuged at 12,000g (15,000 rpm) for 1 min (Biofuge 15, Heraeus Sepatech GmbH, Hanau, Germany). The supernatant was loaded onto a 10% SDS-polyacrylamide gel using the Bio-Rad Mini-Protean II system (Laemmli, 1970).

## RESULTS

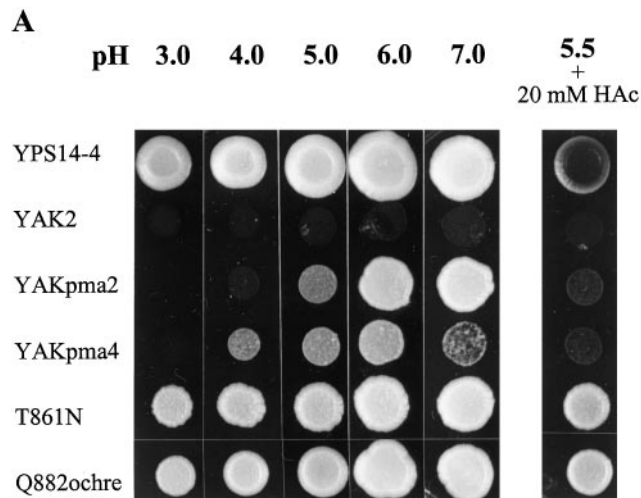
### *pma4* Supports Yeast Growth at Low pH

A cDNA clone for the *N. plumbaginifolia pma4* gene was placed on a yeast 2 $\mu$ -derived multicopy plasmid under the control of the strong and constitutive transcriptional promoter of *PMA1*, the major *S. cerevisiae* H<sup>+</sup>-ATPase gene. The plasmid was introduced into the yeast strain YAK2 deleted from its own two H<sup>+</sup>-ATPase genes, *PMA1* and *PMA2*, and surviving on Gal medium with a centromeric plasmid carrying the yeast *PMA1* controlled by the *GAL1* promoter. Independent transformants obtained in a Gal medium were still able to grow when shifted to a Glu medium, indicating that the plant H<sup>+</sup>-ATPase allowed yeast growth. To eliminate the potential residual expression of yeast *PMA1* under control of the *GAL1* promoter and any recombination between the yeast *PMA1* and the plant *pma4*, we induced the loss of the plasmid bearing the yeast *PMA1* by spreading the transformants on a Glu medium containing 5-fluoroorotic acid, which becomes toxic in the presence of the *URA3* gene present in the latter plasmid. Colonies (YAKpma4) growing in the presence of the drug lost the *URA3* plasmid containing the yeast *PMA1* gene, as confirmed by Southern-blot analysis (data not shown).

The plant H<sup>+</sup>-ATPase PMA4 isoform was thus able to sustain growth of cells devoid of yeast H<sup>+</sup>-ATPase. Moreover, it allowed yeast growth at a pH as low as 4.0 (YAKpma4; Fig. 1A), whereas PMA2, a *N. plumbaginifolia* isoform belonging to the first subfamily, could replace the yeast H<sup>+</sup>-ATPase only if the pH medium was kept above 5.0 (YAKpma2; Fig. 1A). However, at a more alkaline pH (7.0) PMA2 still allowed yeast growth, whereas PMA4 barely allowed growth. These observations were confirmed by measurement of the duplication time in liquid cultures (Fig. 1B). At the optimal pH (6.0), YAKpma2 and YAKpma4 grew 3.4- or 5.3-fold slower, respectively, than a wild-type yeast. At a lower pH, however, YAKpma4 grew better than YAKpma2, confirming its capacity to better sustain a low pH.

### Single Mutations of *pma4* Conferred Growth at Low pH

We recently showed that point mutations in various domains of *N. plumbaginifolia pma2* expressed in yeast improved the H<sup>+</sup>-pumping activity and conferred growth at



**B Duplication time**

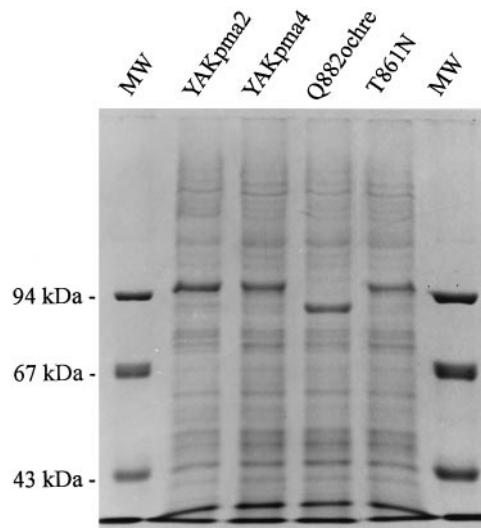
	pH	4.0	5.0	6.0	7.0
YPS14-4		ND	ND	1h44	ND
YAKpma2		∞	10h19	6h05	6h11
YAKpma4		17h33	9h25	9h17	14h44
T861N		ND	ND	4h00	ND

**Figure 1.** Growth at different pH levels of yeast cells expressing wild-type and mutant plant plasma membrane H<sup>+</sup>-ATPases. A, The wild-type YPS14-4 strain (yeast *PMA1* under its own promoter), the recipient strain YAK2 (yeast *PMA1* under the *GAL1* promoter), YAKpma2, YAKpma4, and the mutants T861N and Q882ochre were grown in YGlu medium at pH 6.5 and spotted onto solid YGlu medium at pH 3.0, 4.0, 5.0, 6.0, and 7.0, and at pH 5.5 in the presence of 20 mM acetic acid (HAc). B, The indicated strains were inoculated in liquid YGlu medium at pH 4.0, 5.0, 6.0, and 7.0, and the cell number was scored periodically. The duplication time was calculated during the exponential phase. ND, Not determined; ∞, no division.

pH 4.0 (Morsomme et al., 1996, 1998). Because the wild-type *N. plumbaginifolia pma4* already allowed yeast growth at pH 4.0, we searched for mutants able to grow at pH 3.0. When independent colonies were cultured at a permissive pH (5.5) and streaked on selective plates at pH 3.0, five spontaneous mutants were obtained. The plasmid containing the *pma4* gene from YAKpma4 and each of the selected mutants was retrieved and reintroduced into *E. coli*. The coding sequence of *pma4* in the wild type and each of the mutants was then determined using a series of synthetic primers. In three cases, no modification of the *pma4* gene was found, indicating that genetic modification occurred elsewhere in the plasmid or in the host genome (extragenic mutation). However, a single nucleotide change within the *pma4* gene did occur in another two mutants (intragenic mutation). In the mutant T861N, a point mutation (C to A) transformed the amino acid Thr-861 (ACT) into Asn (AAT), whereas in the mutant Q882ochre, a point mutation (C to T) created a stop codon at codon 882 (CAG to TAG), resulting in an H<sup>+</sup>-ATPase shortened of the last 71 amino acids.

To determine whether the two mutations detected in the plant *pma4* were indeed responsible for improved growth, the retrieved plasmids containing wild-type or mutant plant *pma4* genes were reintroduced into the YAK2 strain, which was then depleted of the centromeric-*URA3* plasmid containing the yeast H<sup>+</sup>-ATPase gene, as described above. The yeast strains containing wild-type and mutant *PMA4* were tested for growth at various pH levels. Unlike YAKpma4, the two mutants grew at pH 3.0 and 7.0 (Fig. 1A). A preliminary interpretation of these data suggested that the mutations that had occurred in T861N and Q882ochre improved H<sup>+</sup>-ATPase functioning. To test this hypothesis, we grew the different strains at pH 5.5 in the presence of 20 mM acetate. This weak acid is thought to acidify the cytosol and prevent the growth of cells containing partially defective H<sup>+</sup>-ATPase that is unable to create a strong electrochemical H<sup>+</sup> gradient across the plasma membrane (McCusker et al., 1987). Only YPS14-4, a wild-type yeast strain expressing its own H<sup>+</sup>-ATPase genes, and the two mutants T861N and Q882ochre were able to grow in the presence of acetate (Fig. 1A), suggesting that their H<sup>+</sup>-ATPases function better.

We have seen so far that the plant *pma4* allows yeast growth at a lower pH than *pma2* and that two *pma4* mutants allow growth at an even lower pH. These different growth properties could be attributed to distinct amounts of H<sup>+</sup>-ATPase assembled in the plasma membrane or to distinct intrinsic enzyme properties. To clarify this point, plasma membranes were purified from the various transformants and the proteins were analyzed by SDS-PAGE (Fig. 2). Plant H<sup>+</sup>-ATPases appeared as the major band in the plasma membrane fraction. Their identity was confirmed by western-blot analysis (data not shown). The



**Figure 2.** Electrophoretic analysis of plasma membrane proteins of yeast cells expressing wild-type and mutant plant plasma membrane H<sup>+</sup>-ATPases. Purified plasma membranes (30 μg) prepared from YAKpma2, YAKpma4, and the two mutants Q882ochre and T861N were analyzed by electrophoresis on a 10% polyacrylamide gel and stained with Coomassie brilliant blue. Molecular mass markers (MW) are shown on the left in kilodaltons.

Q882ochre H<sup>+</sup>-ATPase had a lower apparent  $M_r$  (approximately 90,000), in agreement with the nonsense mutation identified. The pattern shown by this mutant also indicated that the major band observed in the other strains integrally represents the H<sup>+</sup>-ATPase. Considering the H<sup>+</sup>-ATPase amount, no large difference could be identified between the strains, except for a slightly larger quantity for YAKpma2 and Q882ochre. Therefore, better growth performances could not be explained by a larger amount of H<sup>+</sup>-ATPase in the plasma membrane. Therefore, we compared the kinetic properties of the H<sup>+</sup>-ATPase isoforms.

### PMA2 and PMA4 H<sup>+</sup>-ATPases Have Distinct Enzymatic Properties

The apparent  $K_m$  for MgATP and  $V_{max}$  for ATP hydrolysis were determined on a purified plasma membrane fraction (Table I). We observed a higher  $V_{max}$  for PMA4 compared with PMA2, whereas their  $K_m$  values were not significantly different. Because the amount of PMA4 was slightly lower in the plasma membrane fraction (Fig. 2), we conclude that the molecular activity for the PMA4 H<sup>+</sup>-ATPase was clearly increased. A decrease of  $K_m$  and a further increase of  $V_{max}$  was observed for the two mutants derived from YAKpma4 (Table I).

The pH dependence of H<sup>+</sup>-ATPase activity provided another means of distinguishing the isoforms. Both PMA2 and PMA4 had a similar pH optimum (around 6.6–6.8; Fig. 3). However, sensitivity to alkaline pH was much more acute in PMA4. For example, at pH 7.4 the activity of PMA2 was still around 80% of the optimum, whereas that of PMA4 decreased to less than 25%. The pH optima for both PMA4 mutants were not modified but their activity did not decrease as sharply as that of PMA4 at alkaline pH.

LPC is a phospholipid that specifically stimulates plant H<sup>+</sup>-ATPase (Palmgren et al., 1988). Maximal stimulation for PMA2 (about 3-fold) was obtained at 200  $\mu\text{g}/\text{mL}$  LPC (Fig. 4). Stimulation of PMA4 occurred at a lower LPC concentration but decreased above 50  $\mu\text{g}/\text{mL}$  LPC. A more dramatic effect was observed for both mutants; their H<sup>+</sup>-ATPase was only slightly increased at low LPC concentrations and became almost completely inhibited at 200  $\mu\text{g}/\text{mL}$  LPC.

### PMA2 and PMA4 H<sup>+</sup>-ATPases Confer Different Rates of External Acidification

Acidification of the external medium of a yeast suspension has usually been interpreted as a reflection of H<sup>+</sup>-

ATPase activity (Foury et al., 1977; Serrano et al., 1986). Therefore, we recorded the rate of acidification of the external medium induced by the addition of an energy source (Glu). Although Glu might also operate as a regulator of the yeast H<sup>+</sup>-ATPase (Eraso and Portillo, 1994), this is unlikely for the plant enzymes expressed in yeast, because sequences involved in Glu regulation in the yeast pump are not present in the plant enzymes. Despite the similarity in their initial rate, the steady-state level of acidification by the YAKpma4 strain exceeded that of YAKpma2 (Fig. 5). The mutants T861N and Q882ochre performed even better. These in vivo observations are in agreement with the phenotypes observed on solid media at different pH values (Fig. 1).

## DISCUSSION

All of the cDNA encoding for plasma membrane H<sup>+</sup>-ATPases cloned so far from various organs of plant species fall into two subfamilies according to their predicted amino acid sequence identity. Therefore, we can assume that throughout the whole plant these subfamilies account for the major part of the expression of the H<sup>+</sup>-ATPase. It was not possible to determine directly by means of plasma membranes prepared from plant material whether the H<sup>+</sup>-ATPases encoded by these two subfamilies had similar enzymatic properties. Indeed, in *N. plumbaginifolia* (Perez et al., 1992; Moriau et al., 1993), as in tomato (Ewing and Bennett, 1994), transcripts for members of the two subfamilies were found in all of the organs analyzed. However, within an organ, expression might have been restricted to particular cell types, as has already been shown for several genes in Arabidopsis (De Witt et al., 1991; Harper et al., 1994) and *N. plumbaginifolia* (Michelet et al., 1994; L. Moriau, B. Michelet, P. Bogaerts, and M. Boutry, unpublished data). Heterologous expression in the yeast *S. cerevisiae* appeared to be an alternative method for comparing the basic properties of plant H<sup>+</sup>-ATPases.

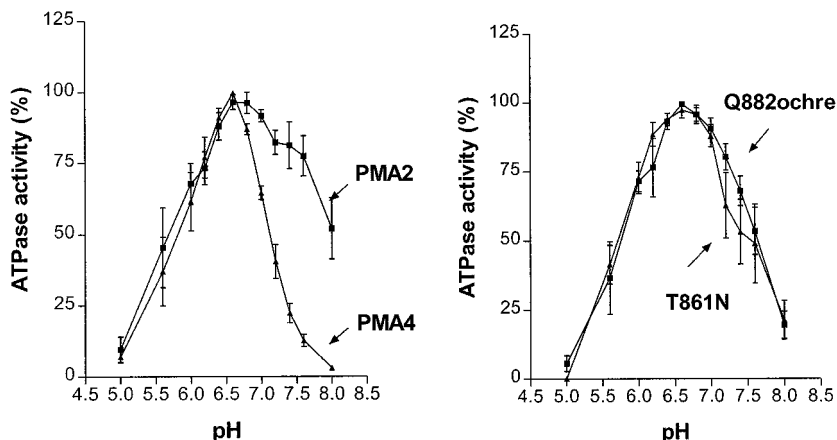
The *pma2* gene, as the most highly expressed from the first *N. plumbaginifolia* subfamily (Perez et al., 1992), had already been characterized in this way (de Kerchove d'Exaerde et al., 1995). Here we have shown that PMA4, the unique member of the second subfamily in *N. plumbaginifolia*, like PMA2, is able to replace the yeast H<sup>+</sup>-ATPase.

The present study provides several lines of evidence demonstrating that PMA2 and PMA4 possess distinct enzymatic properties. Kinetic differences were previously reported for three Arabidopsis H<sup>+</sup>-ATPases, AHA1, AHA2, and AHA3, which were also expressed in yeast (Palmgren and Christensen, 1994). The  $K_m$  for ATP varied between 0.15 and 1.5 mM, and the  $V_{max}$  varied between 1.25 and 2.45  $\mu\text{mol Pi min}^{-1} \text{mg}^{-1}$ . Their pH optimum was similar (6.4–6.5), but AHA3 was more sensitive to inactivation at lower pH values, whereas AHA2 showed a slightly more rapid decrease of activity on the alkaline side of the peak. AHA2 was stimulated by LPC to a larger extent (160%) than AHA1 (60%) or AHA3 (50%) (Palmgren and Christensen, 1994). The differences observed between *N. plumbaginifolia* PMA2 and PMA4 are also related to kinetics, pH profile, and LPC activation. An important observation was that

**Table I.** Kinetics of the wild-type and mutant plant H<sup>+</sup>-ATPases expressed in *S. cerevisiae*

Strain	$K_m$	$V_{max}$
	$\mu\text{M MgATP}$	$\mu\text{mol Pi min}^{-1} \text{mg}^{-1}$
YAKpma2	312 ± 101	0.43 ± 0.14
YAKpma4	246 ± 57	0.75 ± 0.15
T861N	189 ± 41	0.94 ± 0.05
Q882ochre	118 ± 21	1.47 ± 0.30

**Figure 3.** pH dependence of ATP hydrolysis by purified plasma membrane fractions prepared from YAKpma2, YAKpma4, Q882ochre, and T861N measured as described in "Materials and Methods" at the pH values indicated. ATPase activity is expressed as the percentage of maximal activity observed for each strain. The mean specific activities at the optimal pH were: 0.46 (PMA2), 0.60 (PMA4), 0.69 (T861N), and 1.01 (Q882ochre)  $\mu\text{mol Pi min}^{-1} \text{mg}^{-1}$ .



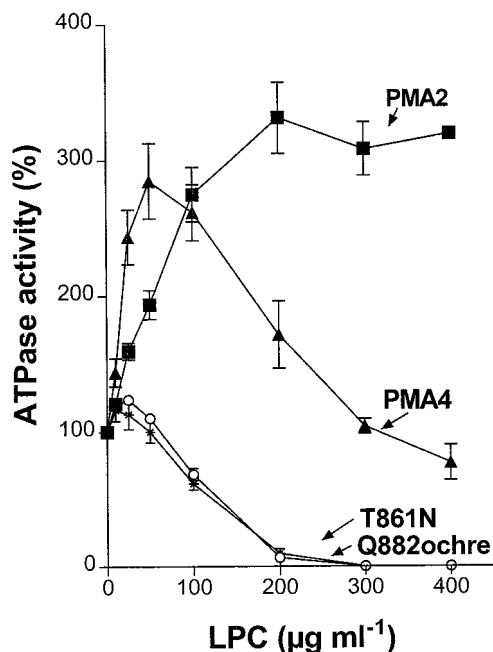
PMA4, like AHA1, AHA2, and AHA3 (all four belong to the same subfamily), showed a sharp decline in activity above pH 7.0, whereas PMA2 had a much broader profile.

At the cellular level, the capacity to allow yeast growth seems to be a major difference between the Arabidopsis and *N. plumbaginifolia*  $\text{H}^+$ -ATPases. Among the three *AHA* genes, only *AHA2* was able to allow yeast growth (to a very low degree) when the yeast  $\text{H}^+$ -ATPase controlled by a Gal-induced promoter was silenced by shifting the cells from a Gal to a Glu medium (Palmgren and Christensen, 1993, 1994). On the contrary, both *N. plumbaginifolia* *pma2* and *pma4* complemented well enough to allow definitive

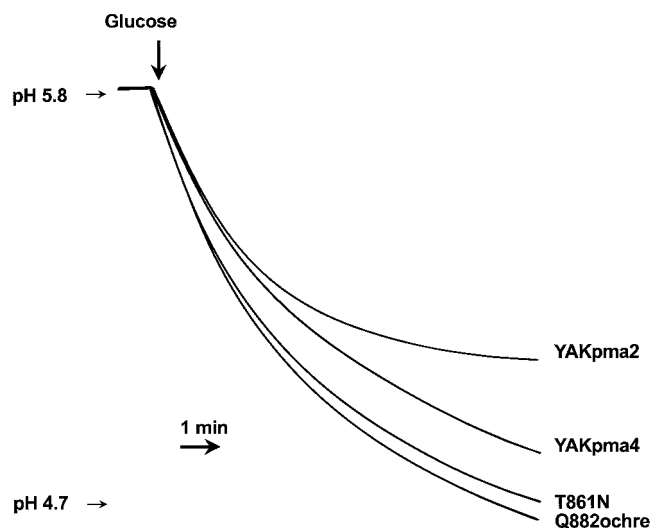
removal of the yeast  $\text{H}^+$ -ATPase gene. The origin of this functional difference is not clear. It does not seem to be explained by differences in ATPase activity, because the Arabidopsis  $\text{H}^+$ -ATPases displayed a 2- to 3-fold higher specific ATPase activity than the *N. plumbaginifolia* PMA2 or PMA4. However, a direct comparison has to be made with care because the Arabidopsis  $\text{H}^+$ -ATPases were characterized in the ER-derived membranes where they accumulate, whereas PMA2 and PMA4 were analyzed in a plasma membrane fraction. A more appropriate comparison would thus require a quantitative analysis of  $\text{H}^+$ -ATPase in the same membrane fraction. A different potential in  $\text{H}^+$ -ATPase isoforms to be directed to the plasma membrane might have a direct consequence on their ability to allow yeast growth. Finally, we cannot exclude the possibility that more trivial factors such as minor differences in the expression system or growth media are responsible for the differences in behavior between the Arabidopsis and *N. plumbaginifolia*  $\text{H}^+$ -ATPases.

The data clearly suggest that PMA4 performs better as an enzyme than PMA2: the  $V_{\text{max}}$  at the optimum pH is higher and this is probably reflected at the cell level by the faster and higher acidification of the external medium. The physiological consequence of this is that *pma4* still allowed yeast growth at pH 4.0, whereas *pma2* did not. This begs the question of whether differences between PMA2 and PMA4 are linked to kinetic or thermodynamic properties. To answer this,  $\text{H}^+$  pumping and the  $\text{H}^+:\text{ATP}$  ratio should be measured. For example, we might consider the possibility that PMA2 and PMA4 differ in their  $\text{H}^+:\text{ATP}$  ratios. A ratio of 1.09  $\text{H}^+$  pumped per ATP hydrolyzed was found for the red beet plasma membrane  $\text{H}^+$ -ATPase (Briskin et al., 1995). However, this ratio might have been an average of data provided by several isoforms expressed in the same organ.

The cells expressing PMA4 grew more slowly at pH 7.0 than at pH 6.0, which was not the case for PMA2. If we assume that in the yeast transformant the pH of the growth medium exerted some influence on the internal pH (Smith and Raven, 1979), the difficulty that YAKpma4 had in growing at pH 7.0 might be explained by the sharp decrease in ATPase activity above pH 6.6 observed for PMA4,



**Figure 4.** Effect of LPC on  $\text{H}^+$ -ATPase activity. ATP hydrolysis was measured as described in "Materials and Methods" on purified plasma membrane fractions prepared from YAKpma2, YAKpma4, Q882ochre, and T861N in the presence of the LPC concentrations indicated, and is expressed as the percentage of activity observed in the absence of LPC. Mean specific activities in the absence of LPC were: 0.44 (PMA2), 0.61 (PMA4), 0.65 (T861N), and 1.14 (Q882ochre)  $\mu\text{mol Pi min}^{-1} \text{mg}^{-1}$ .



**Figure 5.** Acidification of the external medium by yeast cells expressing plant H<sup>+</sup>-ATPase. Cells of YAKpma2, YAKpma4, T861N, and Q882ochre were grown in YGlu medium, washed, and incubated at 30°C for 5 min, and then Glu (250 mM) was added and the pH was recorded.

whereas the activity-versus-pH profile was much broader for PMA2.

A detailed examination of *pma2* and *pma4* expression at the cell level is being developed by means of a reporter gene. Our current observations are that both are expressed in various plant cell types, some of them common to both genes (L. Moriau, B. Michelet, P. Bogaerts, and M. Boutry, unpublished data). If this was confirmed at the protein level, it would indicate that two different isoforms might be found in the same cell. Such a situation might be interesting if a large H<sup>+</sup>-ATPase activity is required for activating intense transport, and also if different H<sup>+</sup>-ATPase isoforms must respond to separate regulatory systems. It has been proposed, and in some cases shown, that H<sup>+</sup>-ATPases are involved in many physiological functions, such as internal pH regulation, nutrient uptake, turgor control, and cell elongation, and are regulated by various endogenous or external factors (Serrano, 1989; Sussman, 1994; Michelet and Boutry, 1995; Palmgren, 1998).

An enzyme such as PMA4, which has a sharp alkaline pH profile, might be well adapted to the regulation of the cytosolic pH (Smith and Raven, 1979). The difference in sensitivity to LPC observed for PMA2 and PMA4 might be another sign of differential regulation. LPC is considered to be a natural regulator because its presence within the plasma membrane may result from the activity of phospholipase A<sub>2</sub> (Palmgren et al., 1988). Phospholipases act within regulatory pathways such as that resulting in the wound response (Lee et al., 1997), and thus might also intervene in the regulatory signaling leading to H<sup>+</sup>-ATPase activation. The reduced stimulation (PMA4) or even the strong inhibition (T861N and Q882ochre) found after increasing the LPC concentration has not been observed for Arabidopsis wild-type or mutant H<sup>+</sup>-ATPase (Palmgren

and Christensen, 1993, 1994). The physiological implications of this are not clear, although it might be an indication of differential regulation of ATPase isoforms. For example, although a low LPC concentration might be involved in displacing the C-terminal region (Palmgren and Christensen, 1993), a high LPC concentration might disrupt some interactions in the hydrophobic region of PMA4 and consequently reduce its activity.

We previously characterized PMA2 mutants that allow yeast to grow at pH 4.0 (Morsomme et al., 1996). Although the YAKpma4 strain could already grow at this pH, the discovery of two *pma4* mutants that grew at pH 3.0 showed that there was still room for improvement. Like the majority of the mutants derived from YAKpma2, the two YAKpma4 mutants were modified in their C-terminal regions. We previously showed that the mutations in *pma2* effectively changed the H<sup>+</sup>-ATPase into an activated conformation, possibly moving away from the C-terminal domain (Morsomme et al., 1998). This presumably also applies to the PMA4 mutants, because the mutation T861N in PMA4 is localized in one residue adjacent to the mutation R865T identified in PMA2 (Morsomme et al., 1996). Moreover, mutants of both enzymes lead to similar changes (e.g. increased enzyme activity, reduced LPC stimulation, and faster acidification of the external medium).

The improved activity of wild-type PMA4 compared with PMA2 could be explained by a change in conformation similar to that suggested for the mutants. Thus, compared with PMA2, PMA4 would be at a more active stage, but would still be subject to improvement by point mutations. The activity difference between PMA2 and PMA4 might also reflect differences in intrinsic properties between these enzymes that are not necessarily relevant to the regulatory C-terminal region.

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