Functional Complementation of a Null Mutation of the Yeast Saccharomyces cerevisiae Plasma Membrane H\(^{+}\)-ATPase by a Plant H\(^{+}\)-ATPase Gene*

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In plants, the proton-pump-ATPase (H\(^{+}\)-ATPase) of the plasma membrane is encoded by a multigene family. The presence within an organ of several isoforms prevents a detailed enzymatic characterization of individual H\(^{+}\)-ATPases. We therefore used the yeast Saccharomyces cerevisiae as a heterologous host for the expression of PMA2, an H\(^{+}\)-ATPase isoform of Nicotiana plumbaginifolia. Yeast transformed by the plant pma2 was still able to grow under conditions where the yeast ATPase gene (PMA1) was either repressed or deleted. The transformed yeast strain was resistant to hygromycin, and its growth was prevented when the medium pH was lowered to 5.0. The N. plumbaginifolia PMA2 expressed in S. cerevisiae has unusual low K\textsubscript{m} for ATP (23 \textmu M) and high pH optimum (6.8). Electron microscopic examination revealed PMA2 in internal structures of the karmelle type which proliferated when cell growth was arrested, either at a nonpermissive pH or at the stationary phase in a minimal medium. Under the latter conditions, subcellular fractionation on sucrose gradients revealed, in addition to the expected plant PMA2 peak linked to the plasma membrane fraction, a low density peak containing PMA2 and KAR2, an endoplasmic reticulum marker. These observations suggest that the partial internal accumulation of PMA2 occurs in membranes derived from the endoplasmic reticulum and largely depends on growth conditions.

Solute transport across the plasma membrane of fungi and plants is powered by a proton motive force built up across the membrane by a proton pump-ATPase (H\(^{+}\)-ATPase). In yeast, H\(^{+}\)-ATPase is encoded by two PMA (plasma membrane H\(^{+}\)-ATPase) genes (Serrano et al., 1986; Schlesser et al., 1988). Only the PMA1 gene, however, is constitutively expressed and essential to growth (Serrano et al., 1986). This simple situation has made it possible to characterize the PMA1 enzyme in detail, using biochemical and genetic approaches (for reviews, see Goffeau and Slayman (1981), Serrano (1989), and Rao et al. (1992)).

The situation in plants is much more complex, since there, H\(^{+}\)-ATPase is encoded by a large family of genes (for reviews, see Sussman (1994) and Michelet and Boutry (1995)). In Nicotiana plumbaginifolia, for instance, nine pma genes have been isolated so far. Four of these have been characterized more extensively and classified in two gene subfamilies according to their homology. All four genes are expressed in most organs of the plant (Perez et al., 1992; Moriau et al., 1993), although expression of each of them is restricted to particular cell types (Michelet et al., 1994).† The occurrence of gene subfamilies might be linked to specialization of the H\(^{+}\)-ATPases, since plant H\(^{+}\)-ATPases trigger a variety of physiological functions (Michelet and Boutry, 1995). It can therefore be hypothesized that, although the basic function of an H\(^{+}\)-ATPase is to couple H\(^{+}\) translocation to ATP hydrolysis, its kinetic parameters and regulatory features might vary according to the isoform. The simultaneous presence of several isoforms within the same organ, however, prevents their individual biochemical characterization. This restriction can be lifted by expressing individual plant pma genes in a heterologous host. Three Arabidopsis thaliana genes encoding the H\(^{+}\)-ATPase isoforms AHA1, AHA2, AHA3 (they are called AHA for Arabidopsis H\(^{+}\)-ATPase) have been expressed in the yeast Saccharomyces cerevisiae (Villalba et al., 1992; Palmgren and Christensen, 1994). However none of these plant H\(^{+}\)-ATPases supported yeast growth when expression of the yeast H\(^{+}\)-ATPase was silenced (Palmgren and Christensen, 1994). This feature prevented the study of the native Arabidopsis ATPase expressed in a still growing S. cerevisiae. Yeast growth occurred, however, when 92 residues of the AHA2 carboxyl terminus, thought to contain a regulatory element, were removed (Palmgren and Christensen, 1993).

In this work, we have analyzed the expression in S. cerevisiae of the pma2 H\(^{+}\)-ATPase from N. plumbaginifolia. This gene was chosen because it belongs to another subfamily than the one comprising the A. thaliana genes aha1–3 and N. plumbaginifolia pma4 gene. The two subfamilies diverged before the separation of current plant families and their divergence might be linked to a specialization of their products (Moriau et al., 1993). In addition, we used a recipient S. cerevisiae strain deleted of its own two H\(^{+}\)-ATPase genes (PMA1 and PMA2) to prevent their recombination with the plant pma2, S. cerevisiae being prone to homologous recombinations that may lead to artefactual data (discussed in Harris et al. (1991, 1993, and

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1 L. Moriau, B. Michelet, M. Oufattole, M. Arango, and M. Boutry, unpublished results.
Strains and plasmids used in this study are described in more detail under "Materials and Methods." Promoter sequences are indicated by a "p" followed by the name in round brackets of the gene from which it is derived.  indicates the fusion of two DNA fragments. Gene names in square brackets denote that these genes are present on a plasmid.

The abbreviations used are: 5-FOA, 5-fluoroorotic acid; ACMA, 9-amino-6-chloro-2-methylmethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; ER, endoplasmic reticulum.

| Strain | Genomic markers | Plasmids | Origin |
|--------|----------------|----------|--------|
| YPS14-4 | MATα, ade-2-101, leu2-211, his3-D200, ura3-52, trp1α63, lys2-801, pmα1::HIS3, pmα2::TRP1 | [PMA1, CEN6, ARSH4, LEU2] | Supply et al. (1993a) |
| YAK1 | MATα, ade-2-101, leu2-211, his3-D200, ura3-52, trp1α63, lys2-801, pmα1::HIS3, pmα2::TRP1 | [PMA1, CEN4, ARS1, URA3] | This work |
| YAK2 | MATα, ade-2-101, leu2-211, his3-D200, ura3-52, trp1α63, lys2-801, pmα1::HIS3, pmα2::TRP1 | [p(GAL1):PMA1] | This work |
| YAKpma2 | MATα, ade-2-101, leu2-211, his3-D200, ura3-52, trp1α63, lys2-801, pmα1::HIS3, pmα2::TRP1 | [p(PMA1):pma2, 2μm, LEU2] | This work |

Plasmid construction (Table I)—The pma2 cDNA was cloned from Nicotiana plumbaginifolia (pma2, Bouttry et al., 1989) was retrieved as a 3,166-bp EcoRI restriction fragment. It starts 18 bp upstream from the initiation ATG and extends 279 bp beyond the stop codon. This fragment was subcloned into the ECoRI site of the centromeric shuttle plasmid pRS-315 (Sikorski and Hieter, 1989). The transcription terminator region of the ADC1 gene was subdivided as a 450-bp BamHI-HindIII fragment (blunted by Klenow DNA polymerase) of plasmid pAAH5 (Ammerer, 1983), into the SalI site (blunted by Klenow DNA polymerase) of pRS-315. Its correct integration was checked by HindIII-SphI restriction mapping.

The HindIII (blunted by Klenow DNA polymerase)-BamHI fragment (934 bp) of plasmid pS15-Pma1 (Supply et al., 1993a), containing the promoter region of the yeast PMA1 gene extending to 3 bp upstream from the transcriptional start codon, was inserted into the EcoRI site of the centromeric shuttle plasmid pRS-315 (Sikorski and Hieter, 1989). This transcription terminator region of the ADC1 gene was subdivided as a 450-bp BamHI-HindIII fragment (blunted by Klenow DNA polymerase) of plasmid pAAH5 (Ammerer, 1983), into the SalI site (blunted by Klenow DNA polymerase) of pRS-315, yielding plasmid p(PMA1)pma2.

The 2-μm multicopy plasmid bearing the pma2 gene was obtained by subcloning the SacI-EcoRV fragment of plasmid p(PMA1)pma2 between the SacI-SmaI sites of the Ycp50 plasmid (Gietz and Sugino, 1988), yielding 2μ(PMA1)pma2.

Plasmid cp(PMA1)pma2 was obtained by inserting the 4.65-kilobase pair HindIII (blunted by Klenow DNA polymerase)-SacI fragment of PMA1 contained in plasmid pECP7Z-PMA1 (Supply et al., 1993a) into the unique EcoRI site (blunted by Klenow DNA polymerase) of the Ycp50 plasmid (Johston and Davis, 1984). The cp(GAL1)PMA1 plasmid was derived from pRS315GPMA1 a pRS-315 plasmid in which the entire coding sequence of PMA1 (the 3.7-kilobase pair Clal-Xbal fragment) (Supply et al., 1993a) is under the control of the GAL1-10 promoter. The pRS315GPMA1 BglII fragment containing LEU2, ARSH4, CEN6, and part of amp' was replaced with the corresponding BglII fragment of pRS-316 (Sikorski and Hieter, 1989), containing URA3, ARSH4, CEN6, and the same part of amp'.

Media—The yeast cells were grown at 30°C on a rich medium containing 2% (w/v) yeast extract (KAT, Ohly, Hamburg, Germany) and 2% of either glucose (YGlu medium) or galactose (YGal medium) or on a synthetic medium containing (per liter): 7 g of yeast nitrogen base without amino acids (Difco), 1.115 g of drop mix (Treco, 1989) supplemented with all the amino acids except those used for selection (histidine, leucine, uracil, tryptophan) and either 2% glucose (MGlucoseLeu,Ura,Trp) or 2% galactose (MGal-His,Leu,Ura,Trp). Solid media contained, in addition, 2% agar (Difco). The 5-FOA medium was prepared as in Treco (1989). When indicated these media were supplemented with 20 mM KH2PO4 and buffered at pH 6.5 (KOH).

Yeast Strains (Table I)—All strains derived from the haploid strain YPS14-4 (Supply et al., 1993a). Yeast cells were transformed according to Ito et al. (1983). The YAK1 strain was obtained as follows: strain YPS14-4 (Ura' ) was transformed with the cp(PMA1)pma2 plasmid. The LEU2 plasmid, also containing the PMA1 gene, was then lost by successive growth cycles on a nonselective leucine-containing medium.

The YAK2 strain was derived from the Δpma2 strain. The latter has the same genotype as YPS14-4 except that the PMA1 gene borne by the pRS-315 plasmid is under the control of the GAL1-10 promoter (pRS315GPMA1). This strain therefore grows only on a galactose-containing medium. The ΔΔpma2 strain was transformed with plasmid cp(GAL1)PMA1 (URA3) and cured of plasmid pRS315GPMA1 (LEU2) by successive growth cycles on a nonselective leucine-containing medium.

The YAK1 strain was transformed with either cp(PMA1)pma2 or 2μ(PMA1)pma2. The YAK2 strain was transformed with 2μ(PMA1)pma2. The control strains were obtained by transformation with the pRS-315 plasmid or the Yeplac181 plasmid with no insert.

Strain YAKpma2 was obtained as follows: Strain YAK2, transformed with 2μ(PMA1)pma2, was plated on MGL-His,Leu,Trp medium at pH 6.5, containing 5-FOA so as to cure the strain of the URA3 plasmid cp(GAL1)PMA1, containing the yeast PMA1 gene. Loss of this plasmid was checked by Southern analysis and Western immunodetection with an antibody raised against the PMA1 protein (Capieaux et al., 1993), pma2 sequencing—The 2μ(PMA1)pma2 plasmid was retrieved

2 The abbreviations used are: 5-FOA, 5-fluoroorotic acid; ACMA, 9-amino-6-chloro-2-methylmethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; ER, endoplasmic reticulum.

3 A. Wach, personal communication.
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...from five colonies of the YAK.pma2 strain and independently transferred to E. coli. Plasmid DNA was prepared and sequenced, using synthetic primers scattered over the pma2 gene.

Plasma Membrane Preparations—Plasma membranes were prepared as in Goffeau and Dufour (1988) with the following modifications. After overnight growth in a 1.25-liter culture, the cells were harvested at a density of either 40–50 × 10^8 cells/ml (synthetic medium) or 100–150 × 10^8 cells/ml (rich medium) and washed three times with ice-cold water. The pellet was divided in two equal parts. One part was resuspended in 2 volumes of 250 mM glucose, the other in 2 volumes of 250 mM sorbitol, and both were incubated for 15 min at 30 °C. After centrifugation, the pellets were resuspended (15 ml/10 g of fresh cells) in either 250 mM glucose or 250 mM sorbitol as sodium addition, 1 mM MgCl₂, 50 mM imidazole adjusted to pH 7.5 with NaOH, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol. The next steps (cell disruption and subcellular fractionation) were unmodified. At the final step, the proteins were resuspended (≥ 1 mg/ml) in 10 mM imidazole, pH 7.5 (NaOH), 1 mM MgCl₂, then aliquoted, frozen in liquid nitrogen, and stored at −80 °C.

The protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin used as a standard.

Subcellular Fractionation by Isopycnic Centrifugation—Subcellular fractionation by isopycnic centrifugation was as developed by Antebi and Fink (1992), with several modifications. Cells were grown in 1 liter of MGlue-His.Leu,Ura,Trp (except for strain YAK.pma2u, grown in MGlue-Ura,Trp, pH 6.5) at a density of 10–15 × 10^8 cells/ml. The cells were converted to spheroplasts by treating them with 0.75 mg/ml deoxycholate dispersion was then added to the plasma membrane suspension (approximately 400 μg of protein), 5 μl of ACMA (0.5 mM stock solution in ethanol), 8 μl of vannilin–acetic acid (10 mM in ethanol), and vanadate (as indicated in Fig. 7). All assay ingredients except MgATP were added and incubated for 7 min at 30 °C. The fluorescence intensity obtained, after correcting for light scattering by the vesicles, by full-scale reading on the recorder was taken as 100%. A 30-μl aliquot of MgATP solution was added to start the reaction (150 mM MgSO₄, 150 mM ATP, 10 mM MES, adjusted to pH 6.9 (strain YAK.pma2) or 6.0 (strain S. cerevisiae) with KOH.

MAGAT-Polyacrylamide Gel Electrophoresis—The SDS-Polyacrylamide Gel Electrophoresis (Laemmli, 1970)—Proteins (65 μg) were suspended in a buffer containing 60 mM Tris/HCl, pH 6.8, 5% (w/v) glycerol, 1% (w/v) SDS, 10 mM dithiothreitol, and 0.005% bromphenol blue. The samples were kept at room temperature for 15 min and centrifuged for 3 min at 12,000 × g (15,000 rpm) in a Biofuge 15. The supernatant was loaded on a 7% polyacrylamide gel (Bio-Rad Protean II for Bio-Rad Mini- and Mini-7 II for a mini-gel). Proteins were stained with Coomassie Blue R-250.

Antiserum Production—Antibodies were raised against synthetic peptides (AHHKSDIERR or TFNELQNLAE) specific to the N. plumbaginifolia PMA2 isoform and purified by affinity chromatography on an ECH Sepharose 4B (Pharmacia) column bearing the immobilized synthetic peptide.

Western Immunoblotting—Samples were fractionated on a 7% polyacrylamide gel as described above. Proteins were blotted (Bio-Rad Trans-Blot system) onto a nitrocellulose membrane in 20% (v/v) methanol, 50 mM Tris, 200 mM glycine, and 0.05% SDS (w/v). Immunodetection was performed as in Harlow and Lane (1988). Yeast PMA1 antisera (Capiaux et al., 1993), yeast KAR2 antisera (Rose et al., 1989), and plant PMA2 antisera against the synthetic peptide TNFELQNLAE were used, respectively, at 6,000−5,000−1,000-fold dilution. 125I-labeled protein A (Amersham, 35 Ci/mg; 100 μCi/ml) was diluted 1,000-fold.

Electron Microscopy—The cells were harvested by centrifugation for 5 min at 6,000 × g, washed with phosphate-buffered saline (20 mM NaH₂PO₄, 140 mM NaCl, pH 7.2), and fixed overnight at 4 °C as a suspension in phosphate-buffered saline containing 4% paraformaldehyde and 0.5% glutaraldehyde. They were then treated with 1% NaOCl and further with NH₄Cl according to Van Tuinen and Riezman (1987). After inclusion in low melting point agarose, the cells were dehydrated with gradient ethanol solutions and embedded in LR white (The London Resin Co., Ltd, Cardiff, United Kingdom). Other sections (0.5 μm) were cut on an ultramicrotome and stained in uranyl acetate (3 min) and lead citrate (3 min). For immunogold labeling the ultrathin sections were labeled with purified polyclonal antibodies against the synthetic peptide AHHKSDIERR and protein A-gold (10 nm) as described by Roth et al. (1978) and stained in uranyl acetate (3 min). To analyze the proliferation of internal membranes, we observed between 100 and 400 sections for each culture.

RESULTS

Expression of the Plant pma2 in Yeast—To avoid artefactual results resulting from homologous recombination events between plant and yeast H^{+}-ATPase genes, we used the haploid S. cerevisiae strain YAK2, lacking the genomic copies of PMA1 and PMA2, the two endogenous H^{+}-ATPase genes. Survival was made possible by the presence of the PMA1 gene under the GAL1 promoter on a URA3-bearing centromeric plasmid. A cDNA clone containing the N. plumbaginifolia pma2 gene was placed under the control of the yeast PMA1 promoter and introduced into a multicopy plasmid additionally bearing the LEU2 selection marker. Independent transformants obtained in galactose medium were able to grow when shifted to glucose medium, demonstrating the ability of the plant H^{+}-ATPase to

4 P. Bogaerts, J.-F. Bridiet, A. Michel, and M. Boutry, unpublished results.
MGlu-His, Leu, Ura, Trp (Glu), 20 mM KH₂PO₄ buffered at pH 5.0 or 6.5.

Serial 10-fold dilutions on solid media MGal-His, Leu, Ura, Trp (Gal) or were grown in MGal-His, Leu, Ura, Trp liquid medium and spotted at membrane H. It is known that yeast cells with a partly defective plasma moter, whereas, in the second culture (16 h), 7% of the cell sections displayed thin, stacked karmellae (Table II; Fig. 2B), whereas at late stationary phase (40 h), 20% of the cell sections showed crumpled, slack karmellae (Table II; Fig. 2C). In all cases, the karmellae were gold-labeled by anti-plant PMA2 (shown in Fig. 2C for cells at late stationary phase).

To examine the density of membranes expressing the plant PMA2, protoplasts were prepared from the three sets of cells described above, and their lysates were submitted to subcellular fractionation by centrifugation on a sucrose gradient. Equivalent material from a control strain without plant pma2, whose growth is prevented in the three growth conditions was tested for comparison.

With cells transferred to the nonpermissive pH, a large peak of ATPase activity was observed between 24 and 46% sucrose (d = 1.0990–1.2079), with a maximum at 36% (d = 1.1562) (Fig. 3A, continuous line). A similar peak was observed when the plant PMA2 was immunodetected, its maximum shifted toward a slightly lower density than the maximum observed with the control strain lacking plant pma2 (Fig. 3A, dashed line and immunodetection). At a permissive pH, however, whether the cells were grown in glucose for 16 h (Fig. 3B) or 40 h (Fig. 3C), two distinct peaks of ATPase activity were seen. One peak appeared at a high density (46%–52%, d = 1.2079–1.2406). For the second ATPase peak (24%–34% sucrose, d = 1.0990–1.1464), its height depended on the growth stage, with approximately four times more ATPase activity after 40 h of growth. Western blot analysis showed a direct correlation between the ATPase activity and the quantity of PMA2 (Fig. 3, B and C). The ATPase activity increased observed in the light peak at late stationary phase paralleled the increase in proliferating internal membranes. This suggests that the low density fraction might contain these membranes, presumably developing from the secretory pathway. To further test this possibility, we immunodetected KAR2, a resident protein of the endoplasmic reticulum lumen (Rose et al., 1989). The distribution of this marker paralleled that of the plant PMA2 in both ATPase peaks. This suggests the higher density peak (46%–52%, d = 1.2079–1.2406) probably corresponds to the endoplasmic reticulum, whose density is close to that of the plasma membrane (Antebi and Fink, 1992).

For the control strain without the plant pma2, which does not grow under these conditions, very little ATPase activity was observed, whatever the culture time on glucose medium (Fig. 3, B and C, dashed line). Interestingly, KAR2 also appeared in the lower density fractions of these cells, after 40 h, indicating that the appearance of this peak does not depend on expression of the plant PMA2.

Subcellular Distribution of PMa2 in a PMA1-null Strain—Once we had established that the plant pma2 could complement the switch off of yeast PMA1 expression (glucose medium), it was important to permanently remove the yeast gene for two reasons. First, residual expression of the yeast PMA1 would be a nuisance during characterization of the plant enzyme. Second, the tendency of S. cerevisiae to undergo homologous recombination might lead to the formation of chimerae between the yeast PMA1 and the plant pma2, this artefact giving rise to incorrect interpretations (Harris et al., 1991, 1993, 1994). We have indeed observed such recombination events between two linearized plasmids, each bearing one of the two genes (de Kerchove d’Exaerde et al., 1995). We therefore spread the YAK2 strain carrying the plant pma2 on a medium at pH 6.5 containing 5-FOA, to cure it of the URA3.
and PMA1-bearing plasmid. As 5-FOA is toxic in the presence of the URA3 gene, colonies growing in the presence of this drug were expected to have lost this plasmid. The absence of the PMA1 gene and of the PMA1 protein was confirmed by Southern and Western blot analysis, respectively (data not shown).

The generation time of this new strain (YAKpma2) was 4 h on both minimal and rich medium. For the control strain expressing yeast PMA1 under its own promoter, the doubling time was 2.5 h on minimal medium and 1.5 h on rich medium. We reproduced with YAKpma2 our previous results showing the dependence of growth on the pH (results not shown). We also tested the strain’s resistance to hygromycin B, an amino-glycoside antibiotic used to select yeast mutants with a partially defective plasma membrane H⁺-ATPase (Mc Cusker et al., 1987). The rationale is that uptake of the drug should be less efficient if a mutation (or, as here, the presence of a heterologous H⁺-ATPase) results in a lower transmembrane electrochemical proton gradient. And indeed, the YAKpma2 strain could still grow in the presence of 300 μg/ml hygromycin, a growth-inhibiting concentration for the control strain (Fig. 1B).

The results of subcellular fractionation and electron microscopy were the same for the PMA1-null strain as for the strain not yet cleared of the yeast PMA1 gene but grown on minimal glucose medium, both strains containing the plant pma2 on a multicopy plasmid; a low density ATPase peak was present (results not shown), and proliferation of karmellae was observed in stationary phase (Table II; Fig. 2D). Gold labeling by anti-plant PMA2 revealed the presence of PMA2 in the proliferating membranes.
To prepare on a large scale membranes intended for enzymatic characterization, we ground the cells with glass beads and used a classic method for plasma membrane purification, i.e. shifting a crude membrane fraction (15,000 g, 340 min) to a pH (pH 5.2) at which mitochondrial and possibly other membranes become aggregated and precipitate while plasma membranes do not (Goffeau and Dufour, 1988). The yeast cells were grown in rich glucose medium (exponential phase), harvested, and, before homogenization, incubated for 15 min in 250 mM glucose, known to activate the yeast H^+-ATPase (Serrano, 1983), or 250 mM sorbitol, used as a control. With cells expressing the yeast PMA1, the specific ATPase activity was increased in the plasma membrane-enriched fraction and activated by incubation with glucose (Table III), but when membranes prepared from the strain expressing only the plant pma2 were used, glucose failed to activate the ATPase. This could be expected since glucose activation has been shown to involve the regulatory carboxyl-terminal region of PMA1 (Portillo et al., 1989), whose sequence is totally different from the corresponding region of the plant PMA2. Another observation was more surprising; the specific ATPase activity of the pma2-expressing strain was lower in the plasma membrane fraction than in the crude membrane preparation. This was not due to an accumulation of PMA2 in internal membranes, since karmellae structures barely arise under the conditions used (exponential growth in a rich medium) (Table II). Gel electrophoresis of the various fractions (Fig. 4A) indicated that the plasma membrane fraction was in fact enriched in plant PMA2 (2.4-fold), albeit less so than the plasma membrane fraction of the yeast PMA1-expressing strain in yeast PMA1 (5.6-fold). We therefore characterized in more detail the plant PMA2, and the yeast PMA1 as a control, in a crude membrane fraction. The electro-
Table III
Specific ATPase activity of membrane fractions from yeast cells expressing yeast PMA1 or pma2

| Strain       | Incubation | Crude membranes | Plasma membranes |
|--------------|------------|-----------------|------------------|
|              |            | μmol min⁻¹ mg⁻¹ protein |                  |
| YPS14-4      | Glucose    | 0.460 ± 0.030    | 2.360 ± 0.370    |
| YPS14-4      | Sorbitol   | 0.330 ± 0.050    | 0.970 ± 0.390    |
| YAKpma2      | Glucose    | 0.153 ± 0.001    | 0.093 ± 0.010    |
| YAKpma2      | Sorbitol   | 0.183 ± 0.004    | 0.092 ± 0.016    |

phoretic analysis (Fig. 4A) also shows that the plant PMA2 migrated faster than the yeast PMA1, although they have similar predicted molecular weight. Western blot analysis with plant PMA2-specific antibodies revealed, in the PMA2-expressing strain (Fig. 4B, lane 3), a band with the same electrophoretic mobility as the band revealed in a plasma membrane fraction isolated from N. plumbaginifolia (lane 2). To rule out the possibility that a mutation occurred in the N. plumbaginifolia pma2 expressed in yeast, we completely sequenced pma2 from the 2µp(PMA1)pm2 plasmid independently retrieved from five YAKpma2 strains. No alteration was found. The different electrophoretic mobility between PMA1 and PMA2 could thus be attributed to post-translational modifications.

Enzymatic Properties of the Plant PMA2—We used a crude membrane fraction (15,000 x g x 40 min) from the yeast cells grown in rich glucose medium (exponential phase), harvested, and, before homogenization, incubated for 15 min in 250 mM glucose, known to activate the yeast H⁺-ATPase (Serrano, 1983), or 250 mM sorbitol, used as a control. The pH optimum was found to be 6.8 (Fig. 5) for the plant PMA2, whether the cells were incubated with glucose or sorbitol. The yeast PMA1 had a more acidic optimum, pH 6.0 (Fig. 5), as already reported (Peters and Borst-Pauwels, 1978; Supply et al., 1993b). Since the cell cultures, membrane preparations, and enzyme assays were performed in parallel, we believe this difference to be significant. It could indicate that at the physiological pH, the plant enzyme is near its maximal activity.

The kinetics of the ATPases was studied in the presence of an ATP-regenerating system, 1 mM free Mg²⁺, and a concentration of MgATP²⁻ varying from 10.3 μM to 4.05 mM. The Michaelis-Menten curves obtained after incubation with glucose or sorbitol were similar. The Eadie-Hofstee plots (Fig. 6, A and B) revealed a similar Kₘ (23.3 and 23.8 μM) and Vₘₐₓ (0.265 and 0.232 μmol of P₁ min⁻¹ x mg⁻¹) in both cases. Interestingly, the first two points of these plots, corresponding to the highest MgATP²⁻ concentrations, did not align with the others, suggesting that the kinetics of the enzyme may be different at high ATP concentration.

Vanadate, a specific inhibitor of cation-transporting ATPases forming a phosphorylated intermediate during their catalytic cycle, inhibited the ATPase activity of the PMA2-expressing strain, with a Kᵣ of 15.2 μM (sorbitol-incubated cells) or 18 μM (glucose-incubated cells) (results not shown). On the other hand, this ATPase activity was stimulated to 169% ± 15% (results not shown) by 300 μg/ml lysophosphatidylcholine, a detergent-like phospholipid known to activate plant H⁺-ATPases in native plant membranes (Palmgren et al., 1988) and when expressed in yeast (Palmgren and Christensen, 1994).

In order to provide final proof that the N. plumbaginifolia PM2 is indeed a proton pump, we studied ATP-dependent proton translocation using purified plasma membranes (Dufour et al., 1982) rescaled by a lecithin/deoxycholate treatment (Venema et al., 1993). The plasma membranes collected after acid precipitation exhibited no ATP-dependent proton-pumping activity (data not shown), but when dispersed with the lysodecin/deoxycholate mix, sealed plasma membrane vesicles were obtained after centrifugation. Interestingly, the ATPase activity lost after acid precipitation of membranes

![Fig. 4. Polypeptide composition of plasma membranes of a yeast strain expressing the plant pma2.](image)

![Fig. 5. pH dependence of ATP hydrolysis by the plant PMA2 and the yeast PMA1.](image)
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**Fig. 6.** Kinetics of the plant PMA2. Crude membranes were prepared from the YAKpma2 strain (expressing only the plant pma2) grown in the rich YGlu medium at pH 6.5 to 120 \(\times 10^6\) cells/ml and incubated in the presence of glucose or sorbitol as described under “Materials and Methods.” The ATP hydrolysis rate was measured in the presence of \([\text{MgATP}]_2\) varying from 0.0013 to 4.05 mm, 1 mm Mg\(^2+\), 10 mm sodium azide, 50 mm ME/SKOH, pH 6.8. An ATP-regenerating system (10 mg/ml pyruvate kinase and 5 mm phosphoenolpyruvate) was added to the mixture. Data are presented as Eadie-Hofstee plots. The slope corresponds to \(-K_m\), and the intercept on the y-axis corresponds to the \(V_{\max}\). The linear regression is the mean of three independent assays (the values at 1.66 and 4.05 mm [MgATP]\(^2\)) were excluded from calculation), the points are the means of the three assays, and the dashed line gives a confidence interval of 95%.

**Fig. 7.** ATP-dependent fluorescence quenching of ACMA in the presence of sealed plasma membrane vesicles from yeast cells expressing only the plant pma2. Sealed plasma membrane vesicles were prepared from strain YAKpma2 (expressing only the plant pma2) and fluorescence quenching measurements were carried out as described under “Materials and Methods,” in the absence (A) or presence (B) of 930 \(\mu\)M vanadate. In A, the release of quenching after addition of carbonyl cyanide p-trifluoromethoxyphenylhydrazone has been corrected for the nonspecific effect of the cation carrier on ACMA fluorescence.

**DISCUSSION**

The key to studying N. plumbaginifolia pma2 expression in yeast was to avoid any yeast H\(^+\)-ATPase expression while enabling the cells to grow. First, the recipient strain was completely deprived of its own two PMA genes to avoid the reconstitution of a functional gene by recombination. Next, since the plant H\(^+\)-ATPase was able to sustain yeast growth, the plasmid-borne yeast PMA1 gene could be removed as well. Expression of the plant PMA2 correlated with the appearance of PMA2-containing karmellae structures. Such membranes were originally observed in yeast cells overexpressing hydroxymethylglutaryl-CoA reductase by Wright et al. (1988), who hypothesized that they derive from the ER. Osmium/potassium ferricyanide fixation, which labels the ER and Golgi membranes (Rambourg et al., 1993), also stained the karmellae structures (data not shown), and the KAR2 protein, an ER marker, co-sedimented with the lower density PMA2-containing membranes proliferating in the stationary phase in a minimal medium. This led us to suggest that this low density fraction contains the karmellae.

The appearance of karmellae does not seem to be linked directly to the level of overexpression of the N. plumbaginifolia PMA2. For instance, we found approximately the same PMA2 level in strain YAK2 harboring 2 \mu p(PMA1)pma2, whether it expressed the yeast PMA1 (galactose medium, stationary phase) or not (glucose medium, stationary phase), yet karmellae were more abundant in the latter case by an order of magnitude. An even greater difference was seen with the strain containing only the plant pma2 gene; although the amount of PMA2 was similar in exponentially growing and stationary phase cells, the karmellae contents were very different: <1%
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(Exponential phase) and 25% (stationary phase). The latter observation concerned cells grown in a minimal medium; growth in a rich medium did not cause proliferation of karmellae. We therefore conclude that overexpression of the plant ATPase is a necessary but not a sufficient condition for karmellae proliferation. When we compare the various situations in which karmellae were observed (Table I), it clearly appears that these structures became abundant when the yeast PMA1 was no longer expressed (after a shift to glucose medium or after removal of the plasmid-borne gene) and, in addition, when the cells were in a minimal medium and not dividing (glucose medium at a nonpermissive pH or stationary phase at a permissive pH). It seems that the karmellae develop when or because the cells are prevented from growing. Although not pointed out by the authors, this conclusion can also be drawn from the results of Villalba et al. (1992) and Palmgren and Christensen (1994) concerning the expression of A. thaliana AHAl, AHA2, or AHA3 H\(^+\)-ATPases (Palmgren and Christensen, 1994), sustain growth of a yeast strain lacking its own PMA genes? Possibly, the pH of the minimal medium used to test AHA complementation was too low. Growth at various pH values has not been reported for these AHA enzymes. Two additional properties, however, distinguish the H\(^+\)-ATPases of the two plant species. One is that N. plumbaginifolia PMA2 has a lower K\(_m\) (23 \(\mu\)M) than AHA1 (150 \(\mu\)M), AHA2 (150 \(\mu\)M), and AHA3 (1.5 \(\mu\)M) (Palmgren and Christensen, 1994), but it is unclear whether this higher apparent affinity of PMA2 for ATP has any physiological consequences in a cell where the cytosolic ATP concentration is usually well above the K\(_m\). A second difference is that the pH optimum of PMA2 is pH 6.8. This means that at the physiological pH, this enzyme is more active than the A. thaliana AHA or S. cerevisiae PMA1 enzymes, whose pH optima are between 6.0 and 6.5. Interestingly, a similar pH optimum was found and complementation was observed in yeast when the A. thaliana AHA2 was truncated of 92 residues at the carboxyl terminus (Palmgren and Christensen, 1993). We checked that no mutation occurred in the plant pma2 borne by the yeast strain depleted of its own PMA1. These results might therefore indicate that the two plant enzymes, which belong to two distinct subfamilies, have different kinetics. As a matter of fact, reporter gene analysis indicates that plant pma genes are differentially expressed according to the cell type and might thus be involved in activating different secondary transport systems (Michelet and Boutry, 1995).

What might cause the lower performance of the plant H\(^+\)-ATPase expressed in yeast? Coupling between H\(^+\) pumping and ATPase activity seems unaltered, since in seeded yeasts the ratio of the H\(^+\) pumping activity (as reflected by the initial rate of ACMA quenching) to the ATPase activity is similar for the strain expressing the yeast PMA1 and the strain expressing the plant PMA2. On the other hand, we could readily compare the amounts of plant PMA2 and yeast PMA1 by staining the plasma membrane proteins of cells expressing one or the other enzyme. The plant PMA2 amounted to approximately 25% of the quantity of yeast PMA1, the other proteins being in similar amount in preparations from both strains. The ATPase activity ratio between the plant and yeast enzymes was more difficult to estimate, because although the purified plasma membrane fraction obtained after acid precipitation was richer in immunodetectable PMA2, it exhibited a lower specific ATPase activity than the crude membrane preparation, possibly because of the acid treatment. Why the PMA2 content is lower is a difficult question. Any level of expression from transcription to protein stability might be involved. Retention of the Arabidopsis H\(^+\)-ATPase in the ER was the proposed explanation for the absence of complementation (Palmgren and Christensen, 1994), but as discussed above, retention of the N. plumbaginifolia PMA2 in ER-derived membranes varies according to the growth stage and growth medium, indicating that retention is rather a secondary effect than due to an intrinsic inability of PMA2 to be brought to the plasma membrane. Alternatively, the fraction of PMA2 that is not correctly targeted might be degraded rapidly in active cells but accumulate in karmellae in cells with a low catabolic activity.

Why could N. plumbaginifolia PMA2, contrary to the A. thaliana AHA1, AHA2, or AHA3 H\(^+\)-ATPases (Palmgren and Christensen, 1994), sustain growth of a yeast strain lacking its own PMA genes? Possibly, the pH of the minimal medium used to test AHA complementation was too low. Growth at various pH values has not been reported for these AHA enzymes. Two additional properties, however, distinguish the H\(^+\)-ATPases of the two plant species. One is that N. plumbaginifolia PMA2 has a lower K\(_m\) (23 \(\mu\)M) than AHA1 (150 \(\mu\)M), AHA2 (150 \(\mu\)M), and AHA3 (1.5 \(\mu\)M) (Palmgren and Christensen, 1994), but it is unclear whether this higher apparent affinity of PMA2 for ATP has any physiological consequences in a cell where the cytosolic ATP concentration is usually well above the K\(_m\). A second difference is that the pH optimum of PMA2 is pH 6.8. This means that at the physiological pH, this enzyme is more active than the A. thaliana AHA or S. cerevisiae PMA1 enzymes, whose pH optima are between 6.0 and 6.5. Interestingly, a similar pH optimum was found and complementation was observed in yeast when the A. thaliana AHA2 was truncated of 92 residues at the carboxyl terminus (Palmgren and Christensen, 1993). We checked that no mutation occurred in the plant pma2 borne by the yeast strain depleted of its own PMA1. These results might therefore indicate that the two plant enzymes, which belong to two distinct subfamilies, have different kinetics. As a matter of fact, reporter gene analysis indicates that plant pma genes are differentially expressed according to the cell type and might thus be involved in activating different secondary transport systems (Michelet and Boutry, 1995). It would be interesting in this regard to test PMA4 of N. plumbaginifolia, for which a cDNA is available and whose close resemblance to A. thaliana AHA1, AHA2, and AHA3 might prevent it from functionally replacing an absent yeast H\(^+\)-ATPase in yeast.

An interesting observation concerning PMA2 kinetics is the unusual behavior of the enzyme at high ATP concentration; the velocity clearly departs from linearity in the Eadie-Hofstee plots, suggesting another kinetic mode at high ATP concentration. This should be further explored. It will be necessary to examine whether a single PMA2 enzyme displays double kinetics or whether two populations of PMA2 with distinct kinetics exist in yeast. The material used in this experiment came from cells grown in a rich medium and displayed a single ATPase peak upon subcellular fractionation on a sucrose gradient. If two populations of PMA2 exist, they must therefore belong to membranes with identical density. Some observations do support the existence of different states of the plasma membrane H\(^+\)-ATPase. For instance, yeast PMA1 is converted to a more active form upon incubation in the presence of glucose (Serrano, 1983), a process probably involving phosphorylation by protein kinases (Chang and Slayman, 1991). The sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, moreover, exists in two states with different affinities for ATP, and conversion from one state to the other also involves phosphorylation (Chambell et al., 1988). H\(^+\)-ATPases from plants such as oat or maize also exhibit complex kinetics (Roberts et al., 1991; Ramos et al., 1994), but these results cannot be unambiguously interpreted as they were obtained with membranes prepared from whole organs which probably express various isoforms. The lowest K\(_m\) observed in this case was 11–16 \(\mu\)M (Roberts et al., 1991), a figure close to that observed for PMA2 in yeast.

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5 L. Moriau, B. Michelet, M. Ouallfate, M. Arango, and M. Boutry, unpublished results.
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In conclusion, we have obtained a \( S. \text{cerevisiae} \) strain whose plasma membrane \( \text{H}^+\text{-ATPase} \) is replaced by the N. \text{plumbaginifolia} PMA2. This has enabled us to characterize biochemically, a single plant \( \text{H}^+\text{-ATPase} \) whose expression permits cell growth. We have shown, moreover, that the trafficking dysfunction revealed in certain circumstances by the proliferation of karmellae structures in this strain depends on the growth medium and growth stage. These observations should enable us to design experiments for testing which growth-related or growth-influencing parameters induce karmellae formation.

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