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Complementation in situ of the yeast plasma membrane H^+-ATPase gene *pma1* by an H^+-ATPase gene from a heterologous species

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In plants and fungi, the transport of solutes across the plasma membrane (pm) is driven by a proton pump (H^+-ATPase) that produces an electric potential and a pH gradient. We expressed *AHA2*, a member of the *Arabidopsis thaliana* pm H^+-ATPase gene family, in yeast cells in which transcription of the endogenous pm H^+-ATPase gene (*pma1*) had been turned off. *AHA2* was expressed mainly in intracellular membranes and only supported very slow growth of transformed yeast cells. Removal of the last 92 C-terminal amino acids from the plant H^+-ATPase produced an enzyme with 2–3-fold higher specific ATPase activity than the wild-type plant enzyme. Surprisingly, the truncated H^+-ATPase was now targetted to the yeast pm and fully supported normal yeast growth.

Proton pump; Regulatory domain; Auto-inhibition; *Arabidopsis thaliana*; *Saccharomyces cerevisiae*

1. INTRODUCTION

The transport of soil nutrients and products of photosynthesis into and within plants is an active process requiring an input of metabolic energy. Solute transport is coupled to cellular metabolism by means of a proton pump (H^+-ATPase; EC 3.6.1.35) embedded within the plasma membrane (pm) [1–3]. This H^+-ATPase generates a pH and electrical gradient across the pm by extruding protons from the cell. The energy bound in this electrochemical gradient provides the driving force for nutrient uptake mediated by solute carriers and channels.

The pm H^+-ATPase has only been observed in plants and fungi. The pm H^+-ATPase of the yeast *Saccharomyces cerevisiae* is thought to act as counterpart to the pm H^+-ATPases in plants [4,5]. However, *AHA1*, a member of the *Arabidopsis thaliana* pm H^+-ATPase gene family, has been introduced into yeast, but the gene product does not support growth of yeast in which transcription of the endogenous pm H^+-ATPase gene (*pma1*) has been turned off [6]. High levels of functional plant pm H^+-ATPase are produced in the transformed yeast cells, but a substantial part of the enzyme remains trapped in the endoplasmic reticulum (ER) [6]. It is therefore possible, that heterologous H^+-ATPases do not functionally complement the yeast H^+-ATPase because they never get to the pm in sufficient amounts.

We expressed the related isoform *AHA2* [7,8] in yeast and found that the gene product could indeed genetically complement *pma1* and support yeast growth, albeit to a very low degree. Because previous work has indicated that an inhibitory domain is located within the hydrophilic C-terminus of the plant H^+-ATPase [9,10] we introduced a stop codon into the coding region of the *AHA2* gene. The mutated cDNA clone, coding for an enzyme lacking 92 C-terminal amino acids, was transformed into yeast. The unexpected result was, that besides having increased activity compared to the wild-type plant H^+-ATPase, a substantial part of the truncated plant H^+-ATPase is found in the pm and the enzyme fully supports normal growth of yeast cells.

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Abbreviations: ER, endoplasmic reticulum; lyso-PC, lysophosphatidylcholine; pm, plasma membrane.

2. MATERIALS AND METHODS

2.1. *Construction of plasmids*

Plasmids pRS-890 and pRS-891 have been described previously [6]. pRS-778 was produced by cloning the *AHA2* cDNA [7] into pBS (Stratagene). *AHA2* was chosen for mutagenesis instead of *AHA1* already expressed in yeast [6] because the coding region of *AHA2* has a convenient *Kpn*I site 0.9 kb to the 3′ end allowing the excision of a fragment suitable in size for cassette mutagenesis [11]. An internal *Sca*I–*Bgl*II fragment of pRS-778 was converted into a *Xho*I–*Xho*I fragment after Klenow treatment and addition of *Xho*I linkers and ligated to the 7.6 kb *Xho*I–*Xho*I fragment of pRS-891 to produce pMP-136. These steps removed a spurious out-of-frame ATG codon 82 bp 5′ to the correct ATG.

2.2. *Mutagenesis*

pMP-132 was constructed by site-directed mutagenesis of pMP-136 according to Ho et al. [11]. The mutagenic oligonucleotide was (changes underlined): T GAG AAC TAG ACG TCT TTC AC corre-

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sponding to the mutation (allele designation between parentheses) $Lys^{856} \rightarrow$ stop (*Δ*aha2-mat6). In addition, the mutagenic oligonucleotide contained a unique *Aat*II site for identification of mutated plasmid in minipreps. Introduction of the correct mutations was confirmed by dideoxy sequencing [12].

2.3. *Yeast strains and culture conditions*

The yeast strains used in this study were: RS-72 [13], RS-933 (RS-72/YEp351 [14]), MP-142 (RS-72/pMP-136), MP-194 (RS-72/pMP-132) and MP-213 (RS-72/pRS-890). The synthetic growth media were as described [6]. In order to express only plasmid-borne enzyme, the galactose-grown cells (absorbance at 660 nm about 1) were diluted 10-fold in glucose medium, and cells were harvested after 24 h.

2.4. *Biochemical methods*

Yeast plasma membranes and endoplasmic reticulum (ER) were purified by differential and sucrose-gradient centrifugation [6]. ATP hydrolysis was measured at room temperature (pH 6.5) in the presence of inhibitors against mitochondrial ATPase, vacuolar ATPase, and acid phosphatase as described previously [15].

SDS-polyacrylamide gel electrophoresis and Western blotting were performed as described [6]. Polyclonal antibodies raised against the carboxyl terminus of yeast H^+-ATPase [16] or against the central part of *Arabidopsis AHA2* [10] were utilized.

3. RESULTS

3.1. *Truncated plant H^+-ATPase fully supports yeast growth*

The entire coding region for one of the *A. thaliana* pm H^+-ATPase genes (*AHA2*) and a mutant of the same gene coding for an H^+-ATPase lacking 92 C-terminal amino acids were subcloned into yeast multicopy plasmids containing the strong promoter of the yeast *pma1* gene. In order to avoid the co-existence of both the yeast and the plant H^+-ATPase in the same cell, we introduced the plant H^+-ATPase expression plasmids into the *S. cerevisiae* strain RS-72 [13]. In this strain, the constitutive promoter of the yeast pm H^+-ATPase gene has been replaced by a galactose-dependent promoter. The resulting transformed strains would express yeast H^+-ATPase on galactose medium but not on glucose medium. The plant H^+-ATPase would be expressed on both media, but at somewhat higher levels on glucose medium. As controls, yeast cells were transformed with the plasmid lacking the cDNA or with plasmid carrying the yeast H^+-ATPase gene.

Expression of functional H^+-ATPase in the yeast pm should support growth of cells devoid of yeast H^+-ATPase, yet yeast cells expressing *AHA2* grew poorly when transferred to glucose medium and formed very small colonies (Fig. 1). On the contrary, yeast cells putatively expressing the truncated H^+-ATPase exhibited normal growth on glucose (Fig. 1). The control strain did not grow at all when transferred to glucose medium.

In liquid minimal glucose medium the growth proper-

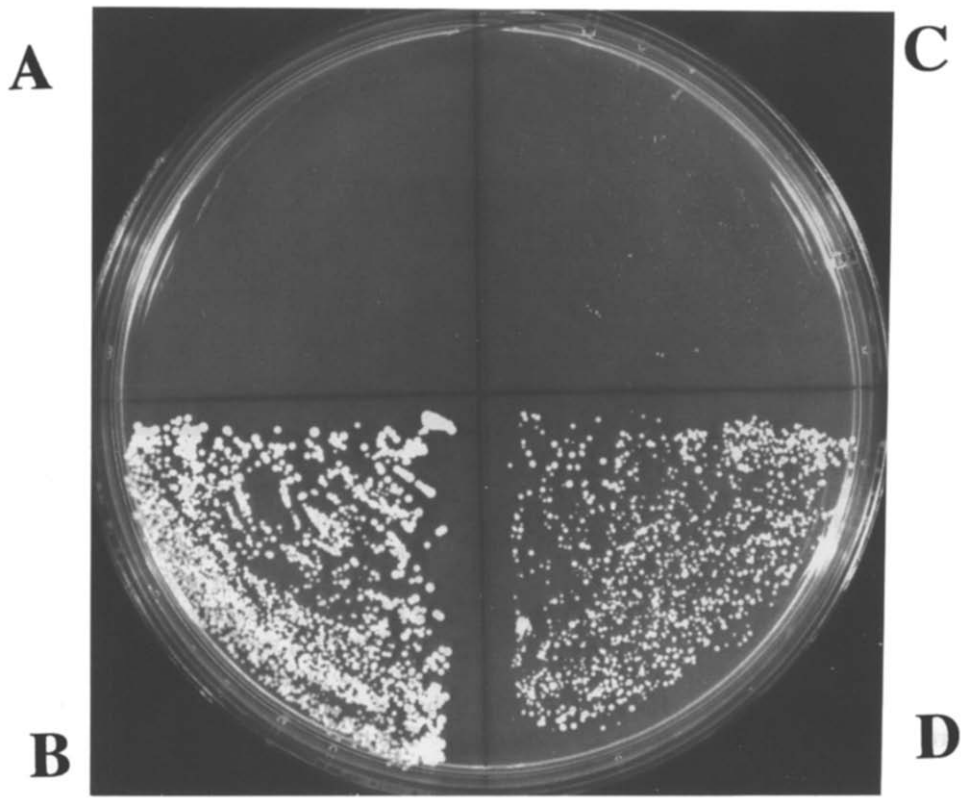

Fig. 1. Test for the growth of yeast strains on glucose medium. (A) Control strain RS-933 (expressing no pm H^+-ATPases). (B) Control strain MP-213 (expressing yeast H^+-ATPase). (C) Strain MP-142 (expressing plant wild-type H^+-ATPase). (D) Strain MP-194 (expressing truncated plant H^+-ATPase). Cells were grown to saturation on galactose medium, and about 10^3 cells were plated on agar plates containing glucose media. Growth was recorded after 3 days.

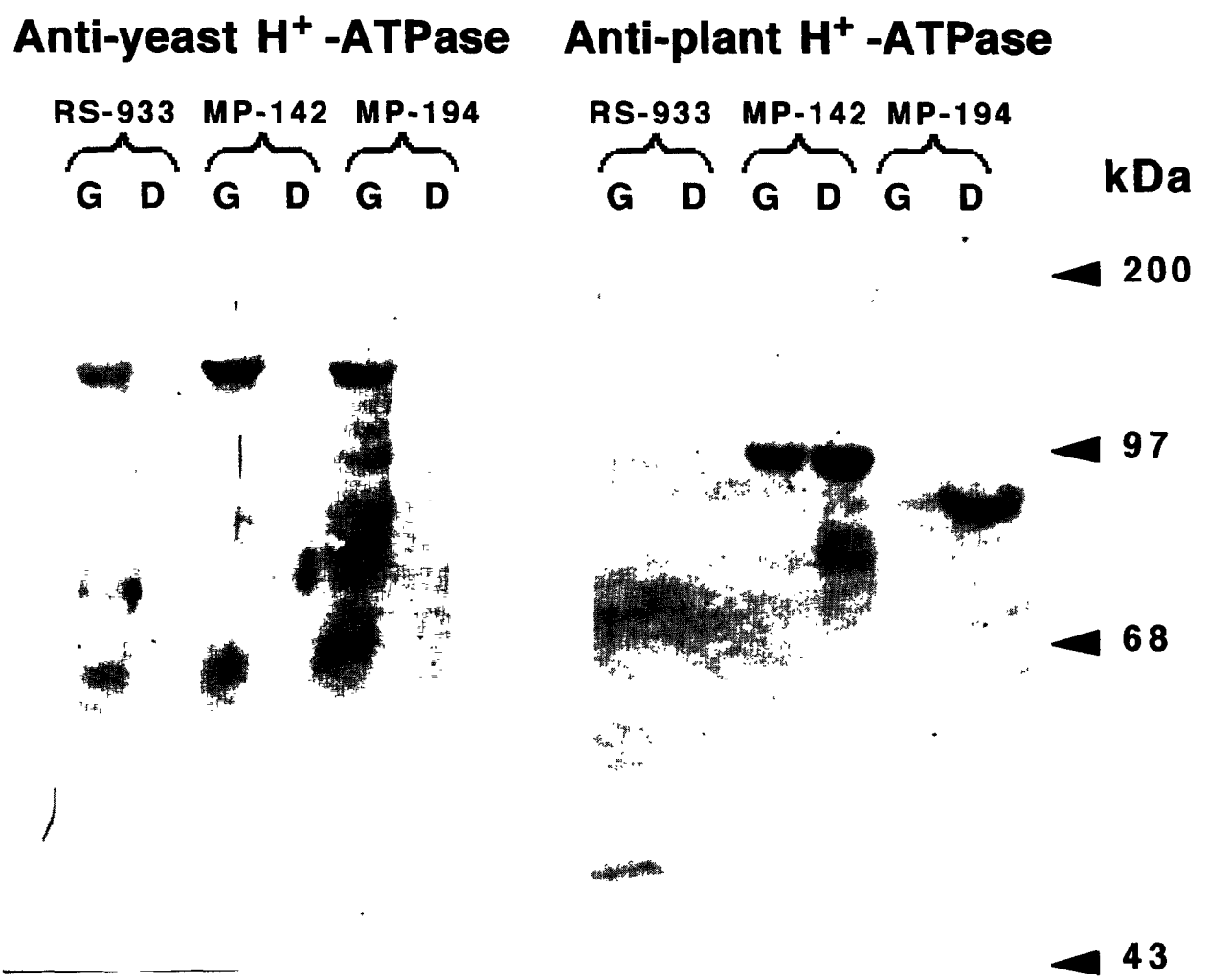

Fig. 2. Expression of plant pm H^+-ATPase protein in yeast. Western blot of total membranes (2 μg of protein per lane and 6% acrylamide gels) from control strain RS-933 (expressing yeast H^+-ATPase only on galactose medium), from strain MP-142 (expressing plant wild-type H^+-ATPase), and from strain MP-194 (expressing truncated plant H^+-ATPase). Strains were cultured in galactose-containing medium until growth reached stationary phase, diluted 100 times with either galactose (G) or glucose (D) medium, and cultured for an additional period of 24 h. Glucose should repress the expression of endogenous yeast H^+-ATPase and boost that of plant H^+-ATPase. The antibodies employed were either against the carboxyl-terminus of yeast H^+-ATPase or against the central domain of the plant H^+-ATPase.

ties of yeast cells expressing truncated plant H^+-ATPase and plasmid borne yeast H^+-ATPase were very similar (duplication times 2.50 h and 3.05 h, respectively). The duplication time of yeast cells expressing wild-type H^+-ATPase exceeded 6 h.

On the basis of immunological detection (Fig. 2), both plant H^+-ATPases were expressed, although the level of wild-type plant H^+-ATPase exceeded that of truncated plant H^+-ATPase (2–3-fold). The level of yeast H^+-ATPase diminished to a level below detection when the cells were transferred to glucose. On the contrary, levels of both plant H^+-ATPases increased 2–3 times in glucose medium as compared to galactose medium. A change in electrophoretic mobility of the truncated plant H^+-ATPase confirms the deletion at the protein level.

3.2. *Truncated plant H^+-ATPase is constitutively activated*

Heterologous plant pm H^+-ATPase expressed in the ER of yeast cells is purified easily and there is no contamination of endogenous yeast ATPases [6]. By quantifying the amount of plant H^+-ATPase polypeptide in the ER and comparing the ATPase activity of the samples (Fig. 3), it was found that the specific activity of the truncated plant H^+-ATPase was 2–3-fold higher than that of wild-type plant H^+-ATPase.

The pm-bound plant H^+-ATPase isolated from plant sources is specifically activated by treatment with the detergent lysophosphatidylcholine (lyso-PC) [3]. Lyso-PC produced a 2-fold increase in the ATPase activity of the wild-type plant H^+-ATPase expressed in the ER (Fig. 4). This increase was not due to the release of

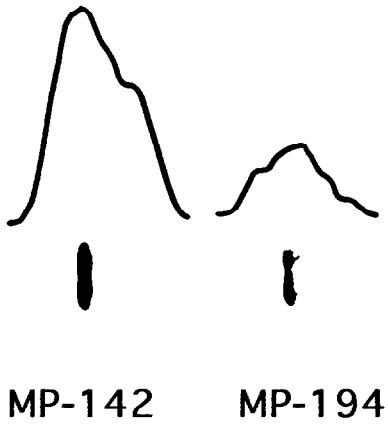

Fig. 3. Correlation between amount of plant H^+-ATPase polypeptide and ATPase activity. ER vesicles were purified from strain MP-142 (expressing wild-type plant H^+-ATPase) and strain MP-194 (expressing truncated plant H^+-ATPase) grown in glucose medium for 24 h. Equal amounts of ATP hydrolytic activity (10 μl membrane preparation; 0.2 μmol P_i released/min/ml) were run on an SDS-polyacrylamide gel and immunoblots were carried out with a polyclonal antiserum against the central part of *AHA2*. The immunoblot shows the region immediately surrounding the H^+-ATPase bands; no other bands were observed. Densitometric scans of the 97 kDa ATPase band in MP-142 and the 85 kDa ATPase band in MP-194 are shown above the immunoblots. The intensity of staining decreased linearly with decreasing amounts of membrane preparation loaded on the gel. No ATPase activity was observed in a similar sample from control strain RS-933.

latent activity since the ATPase activity was not enhanced by other detergents, such as Triton X-100 (Fig. 4) and $C_{14}E_8$ (not shown). In contrast to the wild-type enzyme, the truncated H^+-ATPase expressed in ER was not affected by the addition of lyso-PC (Fig. 5).

3.3. *Subcellular distribution of wild type and truncated plant H^+-ATPase*

When total membranes of yeast cells expressing either wild-type or truncated plant H^+-ATPase were subjected to centrifugation through a linear sucrose gradient, the immunologically detected levels of wild-type plant H^+-ATPase exceeded that of truncated H^+-ATPase (2–5-fold) throughout the gradient (Fig. 5A). A single peak of wild-type plant H^+-ATPase polypeptide could be identified at 30% (w/w) sucrose. This peak corresponds to the novel fraction of enlarged ER [6]. Two peaks of truncated H^+-ATPase could be identified. One peak equilibrated around 30% (w/w) sucrose and a second peak equilibrated at 46% (w/w) sucrose. This latter peak corresponds to the pm, because plasmid-borne yeast plasma membrane H^+-ATPase also equilibrated at 46% (w/w) sucrose (Fig. 5A).

As judged from Coomassie blue staining of membrane proteins separated by SDS-polyacrylamide gel electrophoresis, the truncated plant H^+-ATPase and the yeast H^+-ATPase were expressed to about the same levels in membranes equilibrating at 46% (w/w) sucrose (Fig. 6). The level of wild-type plant H^+-ATPase, indeed, exceeded that of truncated H^+-ATPase and yeast H^+-ATPase 2–3-fold (Fig. 6).

There was little difference in H^+-ATPase activity throughout the gradient between membranes isolated from cells expressing wild-type and truncated plant H^+-ATPases (wild-type: Fig. 5B; truncated: Fig. 5C) despite the differences in amount of plant H^+-ATPase present in the membranes (Fig. 5A). The major peak of ATP hydrolytic activity was found at 30% (w/w) sucrose. This distribution of H^+-ATPase activity corresponded well with that of wild-type H^+-ATPase polypeptide in the gradient (Fig. 5A), but did not match the distribution of truncated H^+-ATPase polypeptide (Fig. 5A).

Latent ATP binding sites facing the lumen of membrane vesicles cannot bind ATP supplied to the extravesicular medium, but may be demasked by addition of a detergent that does not inactivate the H^+-ATPase. In the presence of lyso-PC, a new peak of truncated H^+-ATPase activity appeared at 46% (w/w) sucrose (Fig. 5C), and the distribution of H^+-ATPase activity corresponded well with that of H^+-ATPase polypeptide (Fig. 5A). This effect probably reflects exposure of latent ATP binding sites. The 2–3-fold stimulation of wild-type plant H^+-ATPase activity by lyso-PC throughout the gradient (Fig. 5B) probably reflects the direct activating effect of lyso-PC on this enzyme (see above) suggesting that no or very little ATPase activity is latent.

ER vesicles all expose the catalytic site of the H^+-ATPase to the extravesicular medium and show no latent ATPase activity [6]. Therefore, a substantial

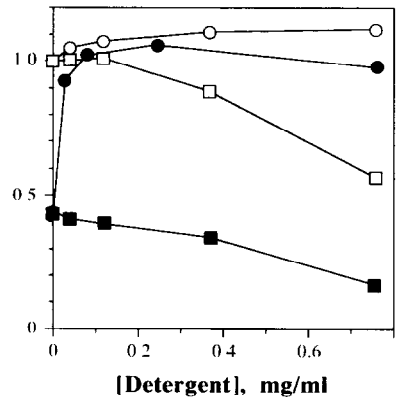

Fig. 4. Effect of lyso-PC (●, ○) and Triton X-100 (■, □) on the ATPase activity of ER vesicles purified from strain MP-142 (●, ■; expressing plant wild-type H^+-ATPase) and strain MP-194 (○, □; expressing truncated plant H^+-ATPase). The amount of plant H^+-ATPase polypeptide in the ER vesicles was quantified in Western blots as described in the legend to Fig. 4. The same amounts of plant H^+-ATPase (2.1 μg of ER protein from strain MP-142; 5 μg of ER protein from strain MP-194) were preincubated with detergents for 5 min in a final volume of 330 μl before addition of ATP. In the absence of detergent, specific ATP hydrolytic activities were 0.82 μmol/min/mg protein (strain MP-142) and 0.78 μmol/min/mg protein (strain MP-194).

amount of wild-type plant H^+-ATPase equilibrating at high densities may result from diffusion of the major ER band at 30% (w/w) sucrose. Alternatively, endomembrane systems containing high amounts of H^+-ATPase polypeptide (e.g. domains of the engorged ER or secretory vesicles) may by chance equilibrate at the same density as the pm. Plasmid-borne yeast pm H^+-ATPase comigrates with the high-density peak of truncated H^+-ATPase polypeptide in the sucrose gradient (Fig. 5A). About 50% of the ATPase activity in purified yeast pm vesicles is latent [17]. We found about 50% latent ATPase activity in membranes equilibrating at 46% (w/w) sucrose containing truncated H^+-ATPase, providing further evidence that these membranes are of pm origin.

4. DISCUSSION

This is to our knowledge the first report demonstrating complementation in situ of the yeast pm H^+-ATPase gene (*pma1*) by an ATPase gene from a heterologous species. An *A. thaliana* pm H^+-ATPase gene, *AHA1*, was previously expressed in yeast but completely failed to complement *pma1*, probably because the gene product was retained in the ER [6]. Using the same expression system we have found that *AHA2*, a related isoform, does indeed genetically complement *pma1*, but still most of the H^+-ATPase polypeptide is trapped in the ER, and the transformed yeast cells grow poorly and form very small colonies. In contrast, a truncated H^+-ATPase, lacking 92 C-terminal amino acids, seems to reach the plasma membrane and completely restores growth of yeast in which transcription of *pma1* has been turned off (Fig. 1). Complementation of *pma1* by *AHA2* implies that the gene products of *pma1* and *AHA2* are functionally interchangeable. It also implies that a mutational analysis of the plant pm H^+-ATPase employing direct in vivo assays in yeast is now possible.

The present work, in addition, provides genetic evidence that the C-terminal region of the plant pm H^+-ATPase constitutes a regulatory domain. Thus, we can confirm the results obtained by a biochemical approach pointing in the same direction [9,10]. The observation that the truncated H^+-ATPase is insensitive towards lyso-PC (Fig. 4) indicates that the C-terminal domain plays a direct role in activation of the H^+-ATPase by this lipid. It is our working model that when the H^+-ATPase needs to be post-translationally activated, the interaction of the C-terminal domain with the ATP binding/catalytic site is exhausted. The release of the constraint may be mediated either by a phosphorylation event in the C-terminal domain [18,19] or by a change in the lipid composition of the pm (e.g., by a rise in PIP_2 [20] or lyso-PC [3]).

The questions remains: since most of the wild-type H^+-ATPase polypeptide appears to remain trapped at a very early stage of the secretory pathway, inserted into the ER, why does truncated H^+-ATPase escape this site of protein synthesis? This question is difficult to answer because very little is known about the molecular machinery that gets integral, hydrophobic proteins from the ER to the pm. It is possible that the C-terminus of the plant H^+-ATPase in some way hinders exit of the H^+-ATPase from the ER or passage of the enzyme through the secretory pathway. Maturation of the yeast pm H^+-ATPase involves phosphorylation at multiple sites during intracellular transport [21]. Thus, phosphorylation may be important for passage of the H^+-ATPase from the ER to the pm via the Golgi apparatus probably by inducing oligomerization and/or association with other proteins. Several lines of evidence suggest that fungal H^+-ATPases exist as hexamers [1]. Oligomers of newly synthesized membrane proteins are recognized by the yeast machinery for 'quality control' before exit from the ER [22]. Since the C-terminus of

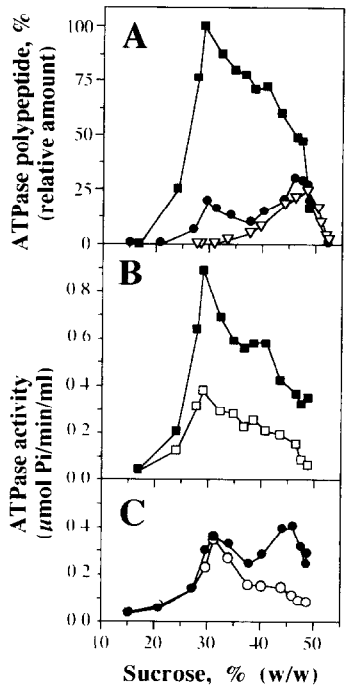

Fig. 5. Distribution of plant H^+-ATPase polypeptide (A) and H^+-ATPase activity (B and C) in sucrose gradient fractions. (A) Total membranes were isolated from yeast grown on glucose medium expressing either wild-type plant H^+-ATPase (strain MP-142; ■), truncated plant H^+-ATPase (strain MP-194; ●), or plasmid-borne yeast H^+-ATPase (strain MP-213; ▽) and loaded on a linear sucrose gradient. After overnight centrifugation, 1 ml fractions were collected from the top of the gradient and used for analysis. Western blots of gradient fractions were stained with antibody against plant or yeast H^+-ATPase polypeptide, and the intensity of staining was quantified as described in the legend to Fig. 3. The staining of yeast and plant H^+-ATPase polypeptides cannot be directly compared since different antibodies were utilized. (B) Distribution of wild-type H^+-ATPase activity in the absence (□) and in the presence of lyso-PC (■) in the same sucrose gradient fractions as in A. (C) Distribution of wild-type H^+-ATPase activity in the absence (○) and in the presence of lyso-PC (●) in the same sucrose gradient fractions as in (A). No H^+-ATPase activity was present in control strain RS-933.

plant H^+-ATPases is about 60 amino acid residues longer than that of the yeast H^+-ATPase [10], it may represent a steric hindrance for proper oligomerization in yeast. Thus, the wild-type plant H^+-ATPase, but not the truncated H^+-ATPase, may form a different kind of oligomer not recognized by the ER export machinery.

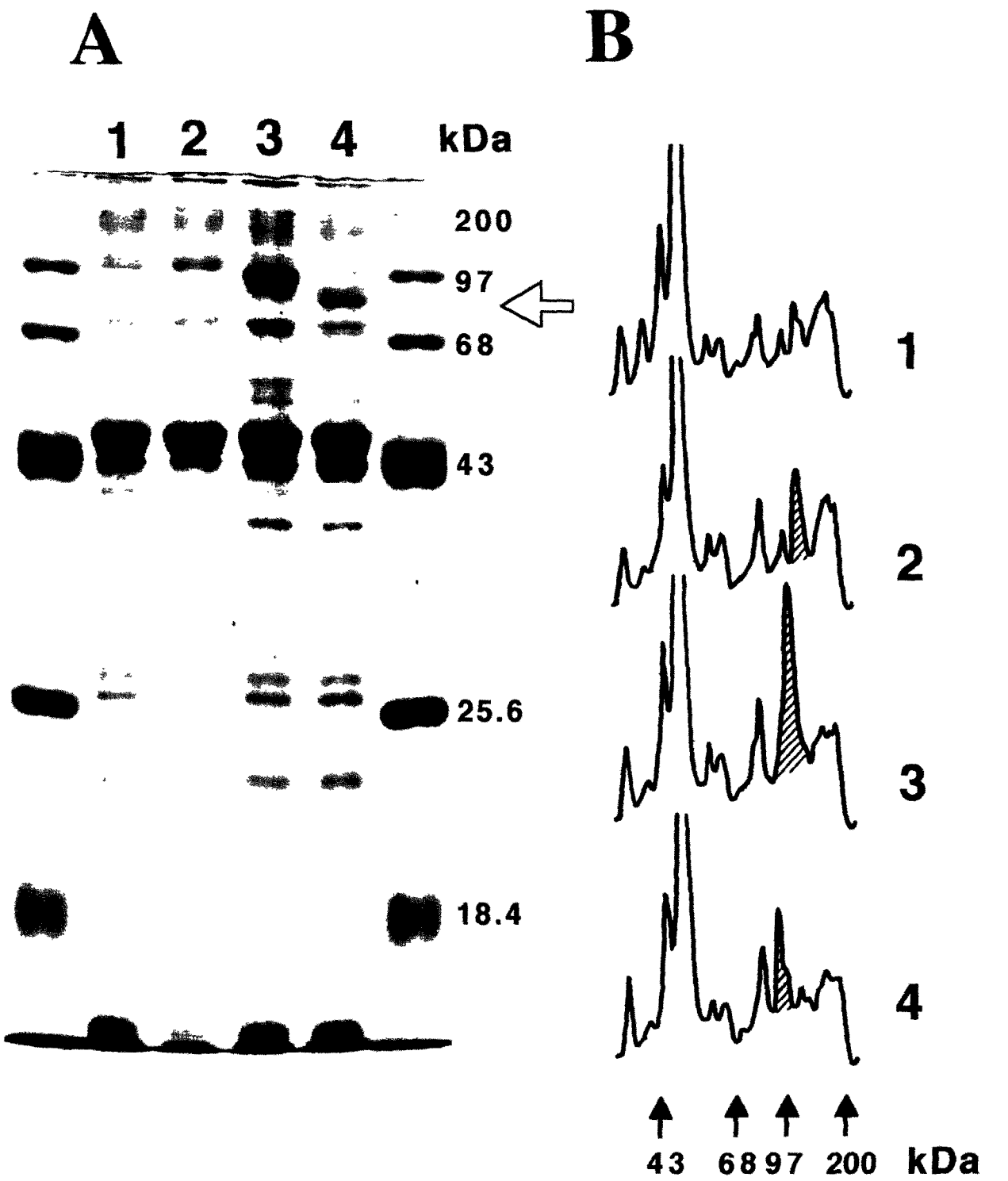

Fig. 6. Polypeptide composition of membranes equilibrating at 46% (w/w) sucrose in a sucrose gradient. (A) Purified membranes (10 μg) from control strain RS-933 (lane 1; expressing no pm H^+-ATPases), control strain MP-213 (lane 2; expressing yeast H^+-ATPase), strain MP-142 (lane 3; expressing plant wild-type H^+-ATPase), and strain MP-194 (lane 4; expressing truncated plant H^+-ATPase) grown in glucose media for 24 h were run on SDS-polyacrylamide gels and stained with Coomassie blue. The position of the truncated plant H^+-ATPase in lane 4 is indicated by an arrow at the right. (B) Relative amounts of high molecular weight polypeptides as visualized by a densitometric scan of the gel shown in (A). The hatched areas correspond to yeast (lane 2) and plant H^+-ATPases (wild-type, lane 3; truncated, lane 4).

It should be kept in mind, however, that at least some wild-type plant H^+-ATPase must be present in the pm, since yeast cells expressing this enzyme did indeed grow. Because expression of wild-type plant H^+-ATPase in yeast is several-fold higher than that of truncated H^+-ATPase, this targeting of enzyme to the pm need not be specific but may reflect a 'spill-over' from the saturated ER. Even if present in sufficient amounts in the pm, the 2–3-fold increase in activity of the enzyme brought about by the C-terminal deletion may be required to increase function beyond the threshold for luxuriant growth.

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