Single Point Mutations Distributed in 10 Soluble and Membrane Regions of the *Nicotiana plumbaginifolia* Plasma Membrane PMA2 H⁺-ATPase Activate the Enzyme and Modify the Structure of the C-terminal Region*

(Received for publication, June 15, 1998, and in revised form, October 5, 1998)

Pierre Morsomme‡, Stéphanie Dambly§, Olivier Maudoux§, and Marc Boutry¶

From the Unité de Biochimie Physiologique, Université Catholique de Louvain, Place Croix du Sud, 2-20, B-1348 Louvain-la-Neuve, Belgium

The *Nicotiana plumbaginifolia pma2* (plasma membrane H⁺-ATPase) gene is capable of functionally replacing the H⁺-ATPase genes of the yeast Saccharomyces cerevisiae, provided that the external pH is kept above 5.0. Single point mutations within the *pma2* gene were previously identified that improved H⁺-ATPase activity and allowed yeast growth at pH 4.0. The aim of the present study was to identify most of the PMA2 positions, the mutation of which would lead to improved growth and to determine whether all these mutations result in similar enzymatic and structural modifications. We selected additional mutants in total 42 distinct point mutations localized in 30 codons. They were distributed in 10 soluble and membrane regions of the enzyme. Most mutant PMA2 H⁺-ATPases were characterized by a higher specific activity, lower inhibition by ADP, and lower stimulation by lysophosphatidylcholine than wild-type PMA2. The mutants thus seem to be constitutively activated. Partial tryptic digestion and immunodetection showed that the PMA2 mutants had a conformational change making the C-terminal region more accessible. These data therefore support the hypothesis that point mutations in various H⁺-ATPase parts displace the inhibitory C-terminal region, resulting in enzyme activation. The high density of mutations within the first half of the C-terminal region suggests that this part is involved in the interaction between the inhibitory C-terminal region and the rest of the enzyme.

The plasma membrane H⁺-ATPase in plants and fungi is an electrogenic pump that couples ATP hydrolysis to proton transport out of the cell. This enzyme is composed of a single type of subunit of about 100 kDa and belongs to the P-type ATPase family characterized by a phosphorylated intermediate during catalysis (1). In the yeast Saccharomyces cerevisiae, H⁺-ATPases are encoded by two genes, PMA1 and PMA2 (2, 3), but only PMA1 is highly expressed and essential. The yeast H⁺-ATPase has been well characterized at biochemical and genetic levels (for reviews, see Refs. 4–7). In plants, on the other hand, H⁺-ATPases are encoded by a multigenic family, the members of which are differentially expressed. An important question to consider is whether all plant H⁺-ATPase isoforms have identical kinetics and whether they are all subjected to similar metabolic regulation. Gene duplication that led to the two most expressed H⁺-ATPase subfamilies predated the divergence of current plant families (8) and might have led to function specialization. Unfortunately, the expression of several isoforms in a same organ (9–11) prevents their individual biochemical characterization and therefore invites the above question.

However, progress has been made through the heterologous expression of plant H⁺-ATPase genes in the yeast S. cerevisiae. Three H⁺-ATPase isoforms of Arabidopsis thaliana belonging to the same gene sub-family (aha1–3) were expressed in *S. cerevisiae* and found to have different kinetics (12). A gene (pma2) from *Nicotiana plumbaginifolia* belonging to another H⁺-ATPase sub-family was also expressed in yeast (YAKpma2 strain). Unlike the *A. thaliana* genes, the *N. plumbaginifolia* pma2 was able to complement the removal of the two yeast H⁺-ATPase genes, provided that the external pH was kept above 6.0 (13). More recently, we selected 21 single point mutants of YAKpma2 that were able to grow at external pH 4.0. The mutations conferred better ATPase and proton-pumping activities on the plant H⁺-ATPase. Most of them were found within the C-terminal region, thus supporting its regulatory role. However, other mutations were located in other regions of the enzyme, thus indicating new residues that are probably involved in regulatory mechanisms (14). These observations invited several questions, such as how many residues are there and which mutation improves the enzyme? Do these mutations have identical effects at structural and functional levels of the H⁺-ATPase?

In this study, we have identified the majority of point mutations able to activate the plant PMA2 H⁺-ATPase expressed in yeast, identifying 10 regions implicated in the regulation of the enzyme. Sixty-three percent of the mutations were localized in two domains within the first part of the C-terminal region, but the others revealed new regions, such as the fourth transmembrane span. Progressive tryptic digestion revealed that the C-terminal region of mutants was more accessible than that of the wild-type, suggesting that the mutations result in a conformational change that displaces the inhibitory C-terminal region.

MATERIALS AND METHODS

Media—Yeast cells were grown either in rich medium, containing 2% (w/v) glucose, 2% (w/v) yeast extract (KAT), and 20 mM KH₂PO₄, or in minimal medium, containing 0.7% (w/v) yeast nitrogen base without amino acids (Difco), 0.115% (w/v) drop mix (15), supplemented by all necessary vitamins. Yeast cells were grown either in rich medium, containing 2% (w/v) glucose, 2% (w/v) yeast extract (KAT), and 20 mM KH₂PO₄, or in minimal medium, containing 0.7% (w/v) yeast nitrogen base without amino acids (Difco), 0.115% (w/v) drop mix (15), supplemented by all necessary vitamins.
amino acids and nucleotides required for growth. Solid media contained 2% agar. The 5-fluoroorotic acid medium was prepared as described in Ref. 15. The medium pH was adjusted with KOH to 6.5 or another pH when indicated.

**Plasmids and Strains**—The plasmid 2μpPMA1/pma2 contains the plant pma2 gene under the control of the *S. cerevisiae* PMA1 promoter (13), the LEU2 gene for selection, and a 2-μm derived sequence for high copy number replication. The control YPS14–4 strain (Mata, ade2–101, leu2Δ1, his3Δ200, ura3–52, trp1–801, lys2–801, pma1-1::TRP1, his3-Δ63, lys2–801, pma1-1::HIS3, pma2::LEU2, pma1-1::HIS3, pma2::HIS3, pma2-2::TRP1) carries the yeast plasma membrane H^+-ATPase PMA1 gene under the control of its own promoter, on a LEU2 centromeric plasmid (16). The YAK2 and YAKpma2 strains have the same genotype as YPS14–4, except that in the former, the PMA1 gene is under the control of the GAL1–10 promoter on an URA3 centromeric plasmid (13), whereas in the latter, the plasmid carrying the yeast PMA1 gene was replaced by the 2μpPMA1/pma2 plasmid (13). Yeast cells were transformed according to Ref. 17.

**Isolation and Sequencing of Mutants**—Independent cultures, each inoculated with a single colony of YAKpma2, were grown at pH 6.5. At stationary phase, 2–3 × 10^8 cells from each culture were streaked on plates containing a rich medium at pH 4.0 (HCl). Spontaneous mutants growing under these nonpermissive conditions appeared after 3 days at 30 °C. To ensure that each isolated mutant was independent, only one mutant per plate was selected. The 2μpPMA1/pma2 plasmid was retrieved from the YAKpma2 or mutant strains and transferred to *Escherichia coli*. The plant pma2 gene was sequenced using 11 synthetic primers dispersed throughout the gene.

**Plasma Membrane Preparations**—Plasma membranes were prepared according to Ref. 18, with the following modifications. After overnight growth in a 1.25-liter culture, the cells were harvested at a density of 50.10^8 cells/ml (rich medium, pH 6.5) and washed three times with ice-cold water. After centrifugation, the pellet was resuspended (15 ml per g of fresh weight) in 250 mM sorbitol, 1 mM MgCl2, 50 mM imidazole-NaOH, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, and the protease inhibitors leupeptin, aprotinin, antipain, pepstatin, and chymostatin at 2 μg/ml. Subsequent steps (cell disruption and subcellular fractionation) were unchanged. In the final step, the proteins were resuspended in 10 mM imidazole, pH 7.5 (NaOH) and 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. (19) using bovine serum albumin as the standard.

**ATPase Assays**—ATPase assays were performed in a 96-hole microplate at 30 °C in a medium containing 2 mM MgATP, 1 mM free Mg^2+ (MgCl2), 50 mM Mes-Mops-Tris (pH 7.0), and 10 mM sodium azide (mitochondrial ATPase inhibitor), as described by Goffeau and Dufour (18), with the following modifications. The reaction was started by adding 40 μl of enzyme (20 μg) to 160 μl of reaction medium. After 3, 6, and 9 min, 50 μl aliquots were taken and mixed with 60 μl 5% trichloroacetic acid to stop the reaction. The optimal pH was determined under the same conditions, with the buffer pH adjusted with HCl or NaOH (from pH 5.0 to 8.0). Inhibition curves were performed in the presence of increasing concentrations of vanadate (from 0 to 200 mM) or ADP (from 0 to 10 mM). Stimulation by lysophosphatidylcholine (LPC) was performed in the presence of increasing concentrations of LPC (from 0 to 800 μg/ml). The specific activity was calculated by linear regression from the slope of the amount of P_i released versus time. The total amounts of ATP and Mg^2+ were added to the reaction mixture at the indicated pH were calculated in order to obtain the desired concentrations of MgATP and Mg^2+ (20).

**Limited Tryptic Digestion**—Limited tryptic digestion was performed at 37 °C on 2 μg of purified plasma membrane fractions with a trypsin/protein ratio of 1/8. The reaction was performed in 50 mM Tris-HCl (pH 8.5) in a final volume of 500 ml. After 0, 0.25, 0.5, 1, 2, 5, and 10 min, 50 μl were taken and added to 10 μl 10% SDS to stop the reaction. After addition of 20 μl of 4-fold concentrated Laemmli’s buffer, 10 μl of each fraction was resolved on standard polyacrylamide gel and transferred to a nitrocellulose membrane. PMA2 fragments were detected by Western blot using polyclonal antibodies directed against either the 110-C-terminal residues, the E118-Q241 fragment (defining the small cytoplasmic loop) of PMA2, or the S305-S620 fragment (defining the large cytoplasmic loop) of PMA2 expressed in *E. coli* as a fusion product with the glutathione-S-transferase (pGEX, Amersham Pharmacia Biotech).

**Results**

Identification of 23 New Mutants of the Plant PMA2—A yeast strain, without its own two plasma membrane H^+-ATPase genes, PMA1 and PMA2, but expressing the *N. plumaginifolia* plasma membrane ATPase pma2 gene, was shown to be able to grow at pH 6.0 but not below pH 5.0 (13). Spontaneous mutants able to grow at more acidic pH had been previously isolated and characterized (14). Twenty-one intragenic mutations mainly localized in the C-terminal region of the plant PMA2 H^+-ATPase were thus identified. This large number of distinct mutated positions led us to wonder whether new regions were still to be discovered. To address this question, we isolated and characterized 40 new intragenic mutants. Among these, we identified 23 new missense mutations (Fig. 1), which revealed 14 new positions in the plant PMA2 not yet identified in the C-terminal region of the plant PMA2 H^+-ATPase. The next step was the construction of a library containing all the intragenic mutants identified in the C-terminal region of the plant PMA2 H^+-ATPase. This library was used to isolate new intragenic mutations.
second one between Arg<sup>779</sup> and Val<sup>855</sup>. Five other mutations were distributed as follows: one nonsense mutation, one deletion, and three duplications of a small sequence, all within the C-terminal region leading either to a frameshift and a shortening of the C-terminal domain or to a longer but modified C-terminal sequence. Twelve mutations corresponded to positions previously discovered. In total, 10 regions of PMA2 were thus affected by point mutations: the N-terminal region, the first transmembrane span, two regions in the small cytoplasmic loop, the fourth transmembrane span, two regions in the large cytoplasmic loop, and three regions in the C-terminal region.

The Mutations Conferred New Physiological and Enzymatic Properties—A common feature of mutants is their ability to grow at acidic pH. This physiological property was previously linked to a better H<sup>+</sup>-pumping activity (14), and this was confirmed for all of the new mutants tested (data not shown). Nevertheless, some mutations could modify in particular some kinetic parameters. To gain an insight into the kinetic parameters, we further analyzed 12 mutants representing each region of the enzyme in which a mutation had been identified by our screening. The ATPase activity of the mutants was either unchanged or increased by up to 2-fold compared with the wild-type plant H<sup>+</sup>-ATPase, except for the P294Q mutant, showing a reduced activity. However, these differences might reflect a higher ATPase content in the plasma membrane or a higher molecular activity. We therefore quantified the PMA2 H<sup>+</sup>-ATPase content after gel electrophoresis on a 10% Tris-Tricine polyacrylamide gel and stained with Coomassie Blue. The band corresponding to the plant H<sup>+</sup>-ATPase is indicated by an arrow. MW corresponds to size markers. The protein bands were quantified using ImageMaster software (Amersham Pharmacia Biotech). The intensity of the band corresponding to the H<sup>+</sup>-ATPase was reported to the total intensity of protein per lane to give the relative H<sup>+</sup>-ATPase amount in the plasma membrane. This value was given as an arbitrary value of 100% for YAKpma2, and the value for the mutants was expressed as the ratio compared with the wild-type PMA2. The data are given in Table I.

### Table I

| Strains     | Specific activity<sup>a</sup> | pma2 quantity<sup>b</sup> | Corrected ATPase activity | LPC activation<sup>c</sup> | pH optimum<sup>d</sup> | I<sub>50 </sub> vanadate<sup>e</sup> | I<sub>50 </sub> ADP<sup>f</sup> |
|-------------|-------------------------------|---------------------------|---------------------------|---------------------------|------------------------|---------------------------|---------------------|
| YAKpma2     | 0.33 ± 0.03                    | 100                       | 0.33                      | 555 ± 13                  | 6.6                    | 11                        | 0.25                |
| E14D        | 0.60 ± 0.07                    | 95 ± 8                    | 0.63                      | 122 ± 47                  | 6.8                    | 12                        | 0.90                |
| P72A        | 0.42 ± 0.04                    | 98 ± 3                    | 0.43                      | 34 ± 9                    | 7.0                    | 80                        | 0.55                |
| W75C        | 0.52 ± 0.06                    | 101 ± 6                   | 0.59                      | 114 ± 10                  | 6.8                    | 11                        | 0.70                |
| P154R       | 0.48 ± 0.05                    | 61 ± 1                    | 0.78                      | 73 ± 38                   | 7.0                    | 11                        | 0.90                |
| H221N       | 0.61 ± 0.08                    | 120 ± 1                   | 0.51                      | 233 ± 12                  | 6.8                    | 7                         | 0.80                |
| P294Q       | 0.17 ± 0.02                    | 158 ± 4                   | 0.11                      | 280 ± 9                   | 6.6                    | 40                        | 0.95                |
| S298L       | 0.48 ± 0.05                    | 66 ± 2                    | 0.73                      | 255 ± 27                  | 7.0                    | 14                        | 1.10                |
| N510K       | 0.52 ± 0.07                    | 73 ± 1                    | 0.71                      | 235 ± 79                  | 6.8                    | 6                         | 1.10                |
| E626G       | 0.54 ± 0.09                    | 122 ± 8                   | 0.44                      | 350 ± 14                  | 7.6                    | 15                        | 0.85                |
| W883C       | 0.44 ± 0.05                    | 62 ± 2                    | 0.71                      | 156 ± 66                  | 7.0                    | 9                         | 0.90                |
| W883L       | 0.37 ± 0.07                    | 65 ± 4                    | 0.57                      | 130 ± 42                  | 7.2                    | 5                         | 0.90                |
| A917V       | 0.62 ± 0.07                    | 70 ± 9                    | 0.88                      | 298 ± 56                  | 7.0                    | 12                        | 0.60                |

<sup>a</sup>Data represent the mean ± S.D. of at least two independent plasma membrane preparations.

<sup>b</sup>The PMA2 amount was quantitated (in duplicate) from Coomassie Blue-stained gel as explained in the legend to Fig. 2.

<sup>c</sup>Values are calculated from a curve obtained from at least two independent experiments.

### Fig. 2

**PMA2 content in plasma membranes from different mutants.** Plasma membrane fractions (10 μg of proteins) were resolved by electrophoresis on a 10% Tris-Tricine polyacrylamide gel and stained with Coomassie Blue. The band corresponding to the plant H<sup>+</sup>-ATPase is indicated by an arrow. MW corresponds to size markers. The protein bands were quantified using ImageMaster software (Amersham Pharmacia Biotech). The intensity of the band corresponding to the H<sup>+</sup>-ATPase was reported to the total intensity of protein per lane to give the relative H<sup>+</sup>-ATPase amount in the plasma membrane. This value was given as an arbitrary value of 100% for YAKpma2, and the value for the mutants was expressed as the ratio compared with the wild-type PMA2. The data are given in Table I.
Mutation and turnover. These changes suggest that the mutants are less active than the wild-type PMA2.

The lack of transmembrane spans affected by mutations indicates that the turnover is not altered by these changes. The mutations that were affected are distributed throughout the protein, but some regions, such as most of the large cytoplasmic loop and the second and third transmembrane spans, are not affected by any mutation. At least two hypotheses could explain this observation. The first is that these regions are not implicated in the catalytic cycle of the H^+-ATPase. The second and not exclusive hypothesis is that some of these regions are involved in the catalytic cycle of the H^+-ATPase. The large loop, for example, is required for the binding of MgATP and the formation of the aspartyl-phosphate intermediate. Mutations in the large cytoplasmic loop would probably affect the turnover and reduce activity. These kinds of mutants were therefore not selected by our screening.

Mutants Reveal New Regions Possibly Involved in PMA2

**DISCUSSION**

**Distribution of the PMA2 Mutations**—We have previously identified 16 PMA2 residues, the mutation of which led to a more effective enzyme (14). In this study, we have identified 14 new positions and 8 new mutations of previously identified positions. Most amino acids involved in the activation of PMA2 made for E14D (Fig. 4) were reproduced for six other mutants analyzed.

**The Mutations Induced a Different Conformation in the C-terminal Region**—LPC activation has been suggested as occurring through the displacement of the inhibitory C-terminal region (22). The reduced LPC stimulation of mutants might indicate that they have already undergone a conformational change that displaced the C-terminal region. To test this hypothesis, we performed limited tryptic digestion on plasma membranes purified from wild-type and mutant strains and we analyzed the H^+-ATPase integrity by means of antibodies directed against either the C-terminal region, the small cytoplasmic loop, or the large cytoplasmic loop. The limited tryptic digestion was performed with antibodies directed against the C-terminal region of the enzyme, disappeared after 5 min for the wild-type PMA2, whereas the degradation was completed between 15 s and 1 min for the mutated PMA2, suggesting that the C-terminal region of the PMA2 mutants contained a more accessible cleavage site for trypsin. In addition to the 100-kDa signal, antibodies against the small cytoplasmic loop detected a ∼90-kDa fragment resulting from the tryptic digestion of PMA2, confirming that the first region to be cleaved in the wild-type as well as in the mutants was the C terminus (Fig. 3, right column) and that the C terminus was cleaved more rapidly in the mutated PMA2 as compared with the wild-type PMA2. The degradation of the 100-kDa band associated with the appearance of the 90-kDa degradation product was also observed with antibodies against the large cytoplasmic loop (not shown).

Protection by vanadate or ADP did not modify sensitivity to trypsin proteolysis (data not shown), indicating that the differences in conformation do not affect the E2 to E1 transition step of the catalytic cycle. However, LPC modified the pattern of degradation for the mutated and the wild-type PMA2 as observed with the anti-C terminus antibodies (Fig. 4). Shorter products were observed in the presence of LPC, suggesting either that LPC protects tryptic sites within the C-terminal region (direct effect) or that LPC changes the H^+-ATPase structure so that tryptic sites within the C-terminal are less accessible (indirect effect). In addition, the profiles of tryptic digests of the mutant and wild-type forms of the plant PMA2 H^+-ATPase in the presence of LPC were very similar even if the degradation was slightly more rapid for the mutant (Fig. 4). Another detergent, Triton X-100, had no effect on wild-type and mutated PMA2 tryptic digestion (data not shown). These results suggest that the differences in conformation between the native and mutated PMA2, observed in the absence of LPC, were almost abolished by this phospholipid. These observations made for E14D (Fig. 4) were reproduced for six other mutants analyzed.

**Mutants Reveal New Regions Possibly Involved in PMA2**

**TABLE**

| Antibody directed against: | C-terminus | Small loop |
|---------------------------|-----------|----------|
| t (min)                   | 0 0.25 0.5 1 2 5 10 | 0 0.25 0.5 1 2 5 10 |
| YAKpma2                   |           |          |
| E14D                      |           |          |
| P72A                      |           |          |
| W75C                      |           |          |
| P154R                     |           |          |
| H221N                     |           |          |
| P294Q                     |           |          |
| S298L                     |           |          |
| N510K                     |           |          |
| E626G                     |           |          |
| W858C                     |           |          |
| W883L                     |           |          |
| A917V                     |           |          |

**Fig. 3**. Limited tryptic digestion of wild-type and mutated PMA2. Purified plasma membranes of the strains expressing the 12 mutated PMA2s, representing each affected region of the H^+-ATPase, and the wild-type PMA2 were digested by trypsin as described under “Materials and Methods” for the indicated times. PMA2 was immunodetected by antibodies directed against the C-terminal region (left column) or against the small loop (right column). With the anti-C-terminal antibodies, we can follow the degradation from the C-terminal region of the full-length enzyme (100 kDa) and with the anti-small loop antibodies, the degradation of the full-length enzyme (100 kDa) associated with the appearance of a 90-kDa degradation product.
Regulation—Most residues affected by mutations are well conserved among plant H^+-ATPases but not in fungal H^+-ATPases (42), suggesting that the mutations involve a regulation mechanism that is specific to plants. As an exception, three mutated residues are conserved in plant and yeast H^+-ATPases: Pro^72, Pro^154, and Pro^724. The last two are also conserved in the rabbit sarcoplasmic Ca^2+-ATPase SERCA1. As a matter of fact, both P72A and P724Q clearly displayed a lower sensitivity to vanadate, whereas P72A and P724R displayed a particular response to LPC (inhibition at high concentration). This suggests that these positions, although involved in regulatory aspects of the plant H^+-ATPase, might also play a role in the basic catalytic activity. In this respect, the structural and functional properties of these mutants might turn out to be different from those of the other mutants, even though they were all selected for their better growth at low pH.

Some PMA2 mutants identified in this study could be compared with mutants of the S. cerevisiae plasma membrane H^+-ATPase (PMA1) and the rabbit sarcoplasmic Ca^2+-ATPase (SERCA1), obtained by directed mutagenesis. Up to now, the N-terminal domain had not been indicated as playing an important role in the yeast PMA1 or mammalian SERCA1 activity, suggesting that the N-terminal region of PMA2, which includes E14D, might be involved in a regulatory mechanism specific to the plant enzyme. Ghu_14 is also the first residue conserved in all plant H^+-ATPases known so far. In contrast, the first transmembrane span of the yeast PMA1 seems to be a very sensitive domain at the structural level. Proline 123 of the yeast PMA1 (scP123) corresponding to the proline 72 of the plant PMA2 (npP72) even though not essential, was suggested as playing a structural role in acting on conformational changes or optimizing the interaction between helices of the yeast PMA1 (24, 25). In the small cytoplasmic loop, the Pro_154 residue of the plant PMA2 is conserved in the yeast PMA1 (scP198) and the mammalian SERCA1 (sercaP147). The scP198A mutation of the yeast PMA1 showed a dominant lethal phenotype (26). The region encompassing the three other mutations within the PMA2 small loop (between Val_220 and His_229) is well conserved among plant and yeast H^+-ATPases. The yeast R271T mutant is dominant lethal (23), and the L275S mutant of PMA1 was found as a second-site revertant of the G158D mutation located in the second transmembrane span (27). The fourth span of both PMA1 and SERCA1 was systematically analyzed by site-directed mutagenesis (28, 29) and contained residues essential for ion transport. The proline conserved in SERCA1 (sercaP236) or PMA1 (scP339) corresponding to Pro_724 in the plant PMA2 largely affected Ca^{2+} transport. From this survey, we can conclude that many PMA2 mutations from the first to the fourth transmembrane spans affect residues that are within or close to regions involved in catalysis and where mutations in the yeast PMA1 and/or the mammalian SERCA1 were deleterious or even lethal. In two cases, the mutations touched a PMA2 residue conserved in the yeast H^- and mammalian Ca^{2+}-ATPase. However, the mutation (P154R or P294Q) improved the plant PMA2 but was deleterious in the other two cases. As the mutations were not the same, this discrepancy could be further investigated by a site-directed mutagenesis interchanging the mutation in each enzyme. The observation that the majority of the PMA1 and SERCA1 mutations had a negative or no significant effect on enzyme activity, whereas the mutations found in the plant PMA2 had a positive effect, emphasizes the significance of the positive selection (yeast growth at low pH) used in this study.

Most Mutations Affect the First Half of the C-terminal Region—The concentration of 65% of the mutations into two segments in the first half of the C-terminal region indicates the critical role of this region in the regulation of plant H^+-ATPases. The C-terminal region is involved in glucose-induced regulation of yeast H^+-ATPase (31, 32), in calmodulin binding in mammalian Ca^{2+}-ATPases (33), and in regulation of plant H^+-ATPases (22). However, the C-terminal sequence is very different in each of these three ATPases, reflecting distinct mechanisms of regulation. Activation of plant H^+-ATPases has previously been induced by in vitro LPC or in vivo fusicioecin treatment (34), or by tryptic digestion of the C terminus (22). Deletions by site-directed mutagenesis within the C-terminal region of A. thaliana H^+-ATPases had the same effect (35). All of these modes of activation led to a slight alkaline shift in the optimum pH and an increase in ATP hydrolysis and proton-pumping activity. These results were generally interpreted as the removal of the inhibitory C-terminal region aside the rest of the enzyme. The mutations identified in this study affecting the C terminus were generally point mutations, but those deletions or insertions in the C-terminal region, modifying the sequence and possibly the structure of the end of the protein. The great majority of the mutations affected the first half of the C-terminal region, possibly resulting in a reduction of or a disruption to the interaction between this region and the rest of the enzyme. This hypothesis is supported by the observation that trypsin had better access to this region in the mutants. Except for A917V, none of the mutations were found in the
Regulation of the Plant Plasma Membrane H\(^+\)-ATPase

second half. This suggests that the first half of the C-terminal region interacts with the rest of the H\(^+\)-ATPase and possibly reduces its activity and that the second half does not participate directly in enzyme autoinhibition. The mutations localized in the other regions of PMA2 might represent areas possibly interacting with the first half of the C-terminal region. The fact that some of these mutations concern residues located within or near predicted transmembrane spans is not surprising if we consider that the mutations also modify H\(^+\)-pumping capacity of the enzyme (14).

**Significance of the Mutations**—All mutations seem to result in a similar structural modification of the PMA2 as shown by the tryptic treatment, thus reflecting a shift between a latent or activated state of PMA2. We therefore suggest that the mutations mimic structural modifications of PMA2 that occur in the plant following a regulatory signal. This signal might well be LPC, a phospholipid known to activate plant H\(^+\)-ATPases (22, 36). In the presence of LPC, trypsin accessibility to PMA2 was modified, and there were no longer any differences between wild-type and mutated PMA2. We therefore suggest that mutations transform PMA2 into its activated form, as does LPC. Moreover, LPC also restricts access to the tryptic sites localized in the first part of the C-terminal region, thus mimicking regulatory modifications of the plant H\(^+\)-ATPase. The availability of a large amount of homogenous latent (wild-type) and activated (mutant) forms of PMA2 should lead to a detailed comparison of their structural and functional properties, thus sidestepping the problem that in plant material, several isoforms coexist, each of which is possibly represented by a mixture of latent and activated forms.

**Acknowledgments**—We would like to thank Bénédicte Purnelle for her help in sequencing and Dr. André Goffeau, Dr. Michel Ghislain, and Vivien Bednarski for their critical reading of the manuscript.

**REFERENCES**

1. Pedersen, P. L., and Carafoli, E. (1987) Trends Biochem. Sci. 12, 146–150
2. Serrano, R., Kielland-Brandt, M. C., and Fink, G. R. (1986) Nature 319, 689–693
3. Schlessel, A., Ulaszewski, S., Ghislain, M., and Goffeau, A. (1988) J. Biol. Chem. 263, 19480–19487
4. Goffeau, A., and Slayman, C. W. (1981) Biochim. Biophys. Acta 639, 197–223
5. Serrano, R. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. 40, 61–91
6. Gaber, R. F. (1992) Int. Rev. Cytol. 37A, 299–353
7. Rao, R., and Slayman, C. W. (1996) in Plasma Membrane and Related ATPases in The Mycota (Brambli, R., and Marzuga, F., eds), Vol. 3, pp. 29–56, Springer-Verlag, Berlin
8. Moriau, L., Bogerts, P., Jonniaux, J.-L., and Boutry, M. (1993) Plant Mol. Biol. 21, 953–963
9. Ewing, N. N., and Bennett, A. B. (1994) Plant Physiol. 106, 547–557
10. Sussman, M. R. (1994) Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 211–234
11. Michte, B., and Boutry, M. (1995) Plant Physiol. 108, 1–6
12. Palmgren, M. G., and Christensen, G. (1994) J. Biol. Chem. 269, 3027–3033
13. de Kerchove d’Exaerde, A., Supply, P., Dufour, J.-P., Bogerts, P., Thines, D., Goffeau, A., and Boutry, M. (1995) J. Biol. Chem. 270, 23828–23837
14. Morsomme, P., de Kerchove d’Exaerde, A., De Meester, S., Thines, D., Goffeau, A., and Boutry, M. (1996) EMBO J. 15, 5513–5526
15. Treco, D. A. (1989) in Current Protocols in Molecular Biology, (Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) pp. 13.11–13.17, Wiley-Interscience, New York
16. Supply, P., Wach, A., Thines-Sempoux, D., and Goffeau, A. (1993) J. Biol. Chem. 268, 19744–19752
17. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 353–368
18. Goffeau, A., and Dufour, J.-P. (1988) Methods Enzymol. 157, 528–533
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
20. Wach, A., Ahlers, J., and Graber, P. (1990) Eur. J. Biochem. 189, 675–682
21. Josephson, L., and Cantley, L. C. (1977) Biochemistry 16, 4572–4578
22. Palmgren, M. G., Sommermair, M., Serrano, R., and larsson, C. (1991) J. Biol. Chem. 266, 20470–20475
23. Portillo, F. (1997) FEBS Lett. 402, 136–140
24. Monk, B. C., Feng, W. C., Marshall, C. J., Seto-Young, D., Na, S., Haber, J. E., and Perlin, D. S. (1994) J. Bioenerg. Biomembr. 26, 101–115
25. Seto-Young, D., Hall, M. J., Na, S., Haber, J. E., and Perlin, D. S. (1996) J. Biol. Chem. 271, 581–587
26. Wang, G., Tamas, M., Hall, M. J., Pascual-Albior, A., and Perlin, D. S. (1996) J. Biol. Chem. 271, 25438–25445
27. Anand, S., Seto-Young, D., Perlin, D. S., and Haber, J. E. (1995) Biochim. Biophys. Acta 1234, 127–132
28. Ambesi, A., Pan, R. L., and Slayman, C. W. (1996) J. Biol. Chem. 271, 22999–23005
29. Rice, W. J., and MacLennan, D. H. (1996) J. Biol. Chem. 271, 31412–31419
30. Vilsen, B., Andersen, J. P., Clarke, D. M., and MacLennan, D. H. (1989) J. Biol. Chem. 264, 21024–21030
31. Portillo, F., de Larrinoa, I. F., and Serrano, R. (1989) FEBS Lett. 247, 381–38
32. Eraso, P., and Portillo, F. (1994) J. Biol. Chem. 269, 10393–10399
33. Carafoli, E. (1994) PASE J. 8, 993–1002
34. Johansson, F., Sommermair, M., and Larsson, C. (1993) Plant Cell 5, 321–327
35. Regenberg, B., Villalba, J. M., Lanfermeijer, F. C., and Palmgren, M. G. (1995) Plant Cell 7, 1655–1666
36. Palmgren, M. G., Sommermair, M., Ulvakov, P., and Jorgensen, P. L. (1988) Physiol. Plant. 74, 11–19
37. Gomes, E., Venema, K., Simon-Plas, F., Milat, M. L., Palmgren, M. G., and Blein, J. P. (1996) FEBS Lett. 386, 48–52
38. Schaller, G. E., and Sussman, M. R. (1988) Plant Cell 17, 503–518
39. Vera-Estrella, H., Barkla, B. J., Higgins, V. J., and Blumwald, E. (1994) Plant Physiol. 104, 209–215
40. Ockinga, C., Pietrowski, M., Hagemeier, J., and Hageman, K. (1997) Plant Cell 10, 411–431
41. Jahn, T., Fuglsang, A., Olsen, A., Bruntrup, I. M., Collinge, D., Volkmann, D., Sommermair, M., Palmgren, M. G., and Larsson, C. (1997) Plant Cell 9, 1805–1814
42. Wach, A., Schlessel, A., and Goffeau, A. (1992) J. Bioenerg. Biomembr. 24, 309–317
43. Xing, T., Higgins, V. J., and Blumwald, E. (1996) Plant Cell 8, 555–564
44. Fullone, M. R., Visconti, S., Marra, M., Fogliano, V., and Aduci, P. (1998) J. Biol. Chem. 273, 7688–7702
Single Point Mutations Distributed in 10 Soluble and Membrane Regions of the Nicotiana plumbaginifolia Plasma Membrane PMA2 H\(^{+}\)-ATPase Activate the Enzyme and Modify the Structure of the C-terminal Region
Pierre Morsomme, Stéphanie Dambly, Olivier Maudoux and Marc Boutry

J. Biol. Chem. 1998, 273:34837-34842.
doi: 10.1074/jbc.273.52.34837

Access the most updated version of this article at http://www.jbc.org/content/273/52/34837

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 42 references, 19 of which can be accessed free at http://www.jbc.org/content/273/52/34837.full.html#ref-list-1