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Transcriptional and post-translational control of the plant plasma membrane H^+-ATPase by mechanical treatments

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Abstract

The activity of the plant plasma membrane (PM) H^+-ATPase was studied with fresh, cut or aged tissues of sugar beet (*Beta vulgaris* L.) leaves. The rate of acidification of the medium by tissue samples was strongly stimulated by ageing, but unaffected by cutting. The proton-pumping activity and the specific activity of the vanadate-sensitive ATPase of purified PM vesicles prepared from aged tissues were much higher than that of fresh tissues, whereas cutting had no effect. Yet, both ageing and cutting increased the amount of PM H^+-ATPase detected by enzyme-linked immunosorbent assays. Likewise, both ageing and cutting increased the levels of pma4 and pma2 ATPase transcripts, as assayed with the corresponding probes from *Nicotiana plumbaginifolia*. Ageing increases, within a few hours, the levels of the transcripts, the translation and the activity of several PM H^+-ATPase families. Cutting, which represents a milder mechanical stress, only increases the levels of the transcripts and their translation, without detectable effect on the activity at the biochemical or physiological level, which suggests a post-translational control of this activity. Thus, upon mechanical stress, the activity of the H^+-ATPase, a key enzyme of the plant PM is rapidly and tightly regulated by transcriptional and post-translational controls.

Keywords: Plasma membrane; ATPase, H^+-; Transcription; Cutting; Ageing; (Plant); (Sugar beet)

1. Introduction

Many experiments pertaining to the area of plant physiology, biochemistry and molecular biology are run with excised (and sometimes aged) tissues. Among many others, transport experiments, in vitro culture, use of suspension cells, of protoplasts, genetic transformation are the most striking examples. It is therefore important to understand the early events following excision which may affect directly the compartmentation of nutrients, and indirectly other physiological processes. The nutrient status of the cell is important for switching on/off various genes [1].

When intact tissues are sectioned and washed, dramatic changes in metabolic activities ensue. Early studies which were devoted mainly to storage tissues, such as carrot roots or potato tubers, characterized these changes as the result of 'washing', also called 'adaptive ageing' as opposite to 'senescent ageing' where a pathway of reduced synthesis leads to an irreversible diminution of physiological competence followed by death [2]. Adaptive ageing (simply referred to as ageing in the following) is obtained by sectioning the tissues into slices, and incubating them for a few hours on either distilled water, or a simple mineral or organic medium. Later studies have shown that ageing also concern leaf tissues, where it induces, among other phenomena, a stimulation of the uptake of organic compounds including hexoses, sucrose and amino acids [3,4].

Excision of a tissue may also affect the energization status of the PM. In barley roots [5] and in extensor tissue of pulvini [6], cutting induces a localized depolarization of the cells close to the injury. This depolarization is due at least in part to H^+ influx into cells around the wound [7]. During ageing, the cells hyperpolarized back to the initial value of PD, or even higher, which suggests that ageing does not only allow a recovery from cutting injury [5]. The hyperpolarization is due to the electrogenic component of the PD [8]. In barley roots, K^+ was also found to affect the hyperpolarization induced by ageing [5].

Abbreviations: CHM, cycloheximide; PD, transmembrane potential difference; PM, plasma membrane; PMV, plasma membrane vesicles; SSC, salt sodium citrate buffer; SSPE, salt sodium phosphate EDTA buffer.

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Given the clearcut effects observed on transport activities, cut and aged tissues represent a useful and convenient model to study the regulation of PM activities. This model is also relevant for many other studies using excised tissues (see above). The increase in the uptake of organic solutes (sugars and amino acids) induced by cutting and ageing may be due to changes in the metabolic activity of the cytoplasm, to changes in the activity of the proton-pumping PM ATPase or to changes in the activity of the transporters mediating the uptake of these organic solutes. Studies with leaf discs or root segments cannot discriminate these different possibilities. Sakr et al. [4] recently compared transport of sugars and amino acids in leaf discs and in PMV from fresh, cut and aged sugar beet leaf tissues. Freshly excised leaf discs were compared with discs that had been floated for 12 h on a simple medium (ageing) and with discs excised from leaves that had been separated from the plant 12 h before the preparation of the discs and the uptake assay (cutting). In all cases, the lower epidermis was removed when the leaf discs were prepared. Ageing induced a general stimulation of uptake (sucrose, hexoses, amino acids), while cutting selectively increased the uptake of sucrose. The physical separation of the tissue from the mother plant therefore induced less dramatic effects than ageing in a large volume of buffer. The selective stimulation of sucrose uptake induced by cutting in leaf discs was also found with purified PMV energized by an artificial proton motive force. It may be concluded that the effects of cutting on sucrose uptake are mediated by membrane transport phenomena, and do not simply result from cell metabolism. Because the energization of the PMV is artificial and do not depend on the proton-pumping ATPase in this system, in vitro stimulation of uptake by cutting into the vesicles cannot be explained by an effect on the ATPase.

However, it is still possible that part of the in vivo stimulation of uptake induced by cutting and ageing in tissues is due to an increase in the activity of the proton-pumping ATPase, with respect to the effects of ageing already described on H^+ fluxes [5–8]. The present work studies the effects of cutting and ageing on the activity and on the expression of the PM proton-pumping ATPase in tissues and PMV from sugar beet leaf.

2. Materials and methods

2.1. Plant material

Conditions for growing the sugar beet (*Beta vulgaris* L.) plants were as described in [9]. The experiments were run with fully expanded leaves. Three kinds of tissues were used: fresh tissues (sampled immediately after separation of the leaf blade from the plant), aged tissues (floated for 12 h in darkness at 20°C on a medium containing 300 mM mannitol, 0.5 mM $CaCl_2$, 0.25 mM $MgCl_2$), and tissues from cut leaves. In the latter case, the leaves were excised from the plant, the extremity (about 2 cm) of their petiole was immersed into distilled water (100 ml for 2 or 3 leaves), and the leaves were kept like this for 12 h in the dark, and saturating humidity, to avoid excessive transpiration. For all series, the lower epidermis was peeled off at the time of disc preparation, to ensure a direct contact between the apoplast and the surrounding medium.

Highly purified PMV were isolated by phase partitioning of microsomal fractions from fresh, cut and aged tissues. Phase partitioning was run in a dextran T500/PEG 3350 mixture as previously described [10].

2.2. Proton extrusion by leaf tissues

Peeled fragments (0.5 g fresh weight, 20 cm^2) from fresh, cut or aged leaves were floated onto 20 ml of a medium containing 300 mM mannitol, 0.25 mM $CaCl_2$ and 0.50 mM $MgCl_2$. The rate of proton pumping was estimated either by monitoring the acidification of the medium or by automatic addition of 1 mM NaOH to maintain the pH at its initial value (5.8) when the tissues started to acidify.

2.3. ATPase activity and proton pumping by purified plasma membrane vesicles

Proteins were assayed according to [11]. ATPase assays were performed at 37°C in a medium containing 25 μg protein, 3 mM ATP, 3 mM $MgSO_4$, 100 mM KCl, 50 mM Tris/Mes (pH 6.5), 250 mM sucrose and 0.02% Brij 58 [12]. After 5, 10, 15, 20 min, successive 100 μl aliquots were sampled and mixed with a stop medium containing 2.1% (w/v) SDS. The specific activity was calculated by linear regression from the slope of P_i release versus time. Various inhibitors were included in the medium, and the pH of the medium was varied accordingly to precise the origin of ATPase activity and the purity of the membrane fractions: 0.25 mM sodium orthovanadate (pH 6.5), 0.1 mM sodium molybdate (pH 6.5), 50 mM sodium nitrate (pH 8.0) or 1 mM sodium azide (pH 8.0).

Proton-pumping was measured by the decrease of amino acridine absorbance at 495 nm in a medium containing 75 μg frozen-thawed PMV, 3 mM ATP, 5 mM valinomycin, 300 mM sorbitol, 10 mM Mops/Bis-Tris propane (pH 6.5), 50 mM KCl, 1 mg ml^{-1} bovine serum albumin and 20 μM acridine orange [13]. The reaction was started by addition of 3 mM (final concentration) of $MgSO_4$ into the medium (1 ml). When the absorbance had decreased to a plateau, 5 μM nigericin (final concentration) were added to the medium.

2.4. Immunological methods

The amount of PM ATPase in the PMV prepared from fresh, cut and aged tissue was estimated by the ELISA

technique already described [14], using anti-PM H^+-ATPase antibodies 758 or 759 as the primary antibody (1/500), and horseradish peroxidase-conjugated goat antirabbit antibody (1/1000) as the secondary antibody. Incubation with the primary and secondary antibody were for 120 and 90 min, respectively. Antibody 758 was raised against the central domain (amino acids 340–650) of isoform 3 of *Arabidopsis thaliana* H^+-ATPase expressed in *Escherichia coli* [15]. Antibody 759 was raised against the C-terminal domain (amino acids 851–949) of the same H^+-ATPase isoform. It is not likely that these antibodies would discriminate between different isoforms of H^+-ATPase [16,17].

For a better solubilization of the PM H^+-ATPase, the procedure of O'Farrell [18] was used in some experiments before adsorbing the PM proteins on the immunoplates. About 1 mg PM protein was pelleted at 29 000 × *g* for 60 min at 3°C, and resuspended in a buffer containing 20% (w/v) glycerol, 4% (w/v) SDS, 2% (w/v) β-mercaptoethanol, 2 mM PMSF and 100 mM Tris, pH 8.5. This suspension was solubilized 3 min at 80°C, and the lipids were sedimented to insoluble material in a Beckman microfuge 15 min at 14 000 rpm. The supernatant was recovered, diluted 4-fold in cold acetone (−20°C) and left at −20°C for 20 min. After centrifugation for 15 min at 15 000 rpm in the microfuge, the pellet recovered was washed with cold 80% acetone, dried under nitrogen and resuspended at room temperature in a buffer containing 9 M urea, 4% Nonidet P40, and 2% β-mercaptoethanol. The solubilized proteins were adsorbed overnight at 4°C on the immunoplates by addition of 1% (final concentration) trichloroacetic acid, and washed by PBS.

In other experiments, the PMV proteins were only treated with 0.1% SDS for 5 min at room temperature, and adsorbed overnight at 4°C on the immunoplates before washing with PBS.

Western blot was performed after separation of the PMV protein by electrophoresis on a 8–12% acrylamide SDS-PAGE gel, and transfer on 0.45 μm pure nitrocellulose membrane (Biorad, Paris), using the antibodies 758 and 759 (1/1000) as the primary antibody, and horseradish peroxidase-conjugated goat antirabbit antibody (1/2000) as the secondary antibody. Incubation with the primary and secondary antibody was for 120 and 90 min, respectively. The efficiency of transfer was checked by the Rouge Ponceau method. Some of the gels obtained after SDS-PAGE were also used for visualization of the protein by silver-staining [9].

2.5. RNA preparation and analysis

The PM ATPase exists as a family of several isoforms encoded by distinct genes. At least two sub-families seem to have diverged before the separation of dicotyledon subclasses and could possibly correspond to enzymes with different regulatory properties [19]. It was thus interesting to study the expression of each of these subfamilies. Northern blot analysis was conducted with RNA prepared from fresh, cut and aged tissues. Probes were cDNA clones for pma2 and pma4, representative of the first and second subfamily of *Nicotiana plumbaginifolia*, respectively. Both genes are expressed in all the plant organs, although at various levels according to the organ and the gene [19,20]. The probes were labeled with [^{32}P]dCTP to a specific activity of about 5 to 8 10^8 cpm μg^{-1} by the random priming technique with the Ready to Go DNA labeling kit (Amersham France, Les Ulis).

Total RNA was prepared by a method modified from Logemann et al. [21]. Briefly, the tissues (2 g) were snap frozen in liquid nitrogen and ground in a mortar cooled with liquid nitrogen. When a fine powder was obtained, 6 ml of grinding medium (8 M guanidinium hydrochloride, 20 mM Mes, 20 mM EDTA, pH 7.0) and 100 μl β-mercaptoethanol were added. The resulting brei was extracted with an equal volume of phenol/chloroform/isoamylic alcohol (25:24:1). After centrifugation (20 min, 10 000 × *g*), the aqueous phase was recovered and added with 0.05 volume of cold 1 M acetic acid and 0.7 vol. of cold absolute ethanol. The RNA were precipitated for 30 min at 4°C and recovered after centrifugation. The pellet was washed twice with 3 ml of 3 M sodium acetate (pH 5.2) to remove the carbohydrates and the glycoproteins. The pellet was then washed with 70% ethanol. After centrifugation, the final pellet was resuspended in 10 mM Tris/1 mM EDTA (pH 8.0) or in diethylpyrocarbonate-treated water, and stored at −70°C.

RNA samples were denaturated for 15 min at 55°C in 2.2 M formaldehyde/50% formamide, and fractionated on 1.1% agarose/2.2 M formaldehyde gels using a Mops buffer (100 mM Mops (pH 7.0), 40 mM Na acetate, 5 mM EDTA (pH 7.0)) [22]. After electrophoresis, the RNA were blotted overnight by capillarity onto a 0.45 μm nylon transfer membrane (Amersham France, Les Ulis). The transfer buffer was 10 × SSPE (1.5 M NaCl, 100 mM $NaHPO_4$, 10 mM EDTA, pH 7.7). The quality and quantity of RNA blotted on membranes was checked by UV absorption. After washing (3 × 2 min in 2 × SSPE), the RNA were fixed to the membrane by heating at 80°C for 2 h. Prehybridization was run for 8 h at 42°C in a medium composed of 6 × SSC (90 mM NaCl, 12. 5 mM Na citrate, pH 7.0), 50% formamide, 2 × Denhardt's solution and 25 $\mu g\ ml^{-1}$ salmon sperm DNA [22]. Hybridization with the ATPase probe was run in the same medium added with the labelled probe for 16 h at 42°C. After hybridization, the membranes were washed twice (2 × 15 min) in 1 × SSC and 0.1% SDS at 50°C.

3. Results

Fig. 1 shows the evolution of the pH of a medium containing peeled fragments from fresh, cut or aged tis-

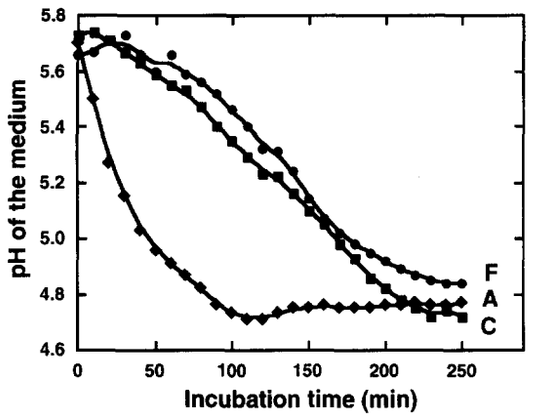

Fig. 1. Time course of the pH of a medium containing fresh (F), cut (C) or aged (A) sugar beet leaf fragments. The data are representative of experiments repeated with six different samples for each treatment.

sues. Fresh and cut leaves acidify the medium at the same rate, from pH 5.8 down to pH 5.2 within 180 min. In both samples, the acidification continued down to a plateau of 4.6–4.7 after 5 or 6 h of incubation. In broad bean, this type of acidification has been shown to be related to proton extrusion, and not to the release of organic acids [23]. Likewise, in the present experiments with sugar beet, addition of the uncoupler carbonyl-cyanide-*m*-chlorophenylhydrazone (10 μM) to the medium when the pH had reached 4.7 resulted in an alkalinization up to pH 6.5 (data not shown), also suggesting that the acidifying activity of the tissues is due to proton pumping. In a medium containing aged tissues (Fig. 1, lower curve), the acidification proceeded immediately at a much higher rate than the rate observed for fresh or cut tissues. Although the plateau of acidification is attained more rapidly, it is not lower than that measured with fresh or cut tissues (pH 4.8). The rate of acidification measured by an automatic titrator maintaining the pH at 6.00 with 1 mM NaOH was 15.0 ± 0.2 nEq H^+ min^{-1} g^{-1} FW (means of 12 samples $\pm$ S.E.) for fresh tissues. Compared to this control value (100%), acidification rates represented $114 \pm 26\%$ ($n = 6 \pm$ S.E.) and $178 \pm 21\%$ ($n = 6 \pm$ S.E.) in tissues from cut and aged leaves, respectively. This confirms that ageing strongly promoted the acidifying activity of the tissues, while cutting had little effect.

Various ATPase activities were measured on PM purified by phase partitioning from fresh, cut and aged tissues. Table 1 shows that azide-sensitive, nitrate-sensitive and molybdate-sensitive ATP-hydrolyzing activities are absent or small compared to the vanadate-sensitive ATPase activity, showing the same high purity for PM preparations obtained from the different samples. The specific activity of vanadate-sensitive ATPase was the same in PM from fresh tissue and cut tissue (541.7 ± 109 and 510.2 ± 15.6 nmol P_i min^{-1} (mg protein)$^{-1}$, respectively), whereas it doubled in PM from aged tissues (1134 ± 216.6 nmol P_i min^{-1} (mg protein)$^{-1}$).

Because activation of the PM H^+ ATPase by proteolysis or fusicoccin in plants [24], and by glucose in yeasts [25] affects the pH sensitivity of the enzyme, it was interesting to compare the pH sensitivity of vanadate-sensitive activity in PMV from fresh or aged tissues. The pH of the incubation medium was varied between 5.4 and 8.0 using Tris/Mes buffers. Both with PMV from fresh and aged tissues, the optimal pH for ATPase activity was around 6.2, and the pH sensitivity observed was quite similar (Fig. 2A).

Various isoforms of the PM H^+-ATPase of yeast [25] and plants [16] may differ by their kinetic parameters. Proteolysis also affects the kinetic parameters of the plant PM H^+-ATPase [24]. Fig. 2B shows that ATPase activity of PM from fresh and aged tissues followed hyperbolic reactions. Ageing increased the V_{max} of the reaction (50% to 150% depending on the experiments), while no clearcut effect was found for the apparent K_m of the enzyme for ATP (Fig. 2B, and data not shown).

Proton pumping activities were measured on series of PMV prepared the same day from fresh and cut tissue. The rate of proton-pumping was 0.18 ± 0.04 OD units min^{-1} (mg protein)$^{-1}$ in PMV from control leaves, and 0.20 ± 0.05 OD units min^{-1} (mg protein)$^{-1}$ in PMV from cut leaves ($n = 14 \pm$ S.E. for each series). Parallel experiments were also run with PMV prepared the same day from fresh leaves and from aged leaves. The rates of proton pumping were 0.19 ± 0.05, and 0.42 ± 0.07 OD units min^{-1} (mg protein)$^{-1}$ in PMV from fresh leaves, and aged leaves, respectively ($n = 7$ for each series). Therefore, ageing more than doubled proton-pumping activity, whereas cutting had no effect on this activity.

Table 1
Purity of PMV prepared from fresh, cut and aged leaves as estimated by the sensitivity of ATPase activity to various inhibitors

	ATPase activity (% of control)		
	Fresh leaves ($n = 8$)	Cut leaves ($n = 4$)	Aged leaves ($n = 4$)
Vanadate-sensitive ATPase	88.3 ± 2.3	86.8 ± 0.8	93.7 ± 3.4
Nitrate-sensitive ATPase	4.9 ± 1.7	3.0 ± 1.2	3.2 ± 2.0
Azide-sensitive ATPase	0.6 ± 0.7	2.5 ± 1.5	0.8 ± 0.5
Molybdate-sensitive activity	5.3 ± 0.3	7.8 ± 3.2	3.1 ± 2.2

Total activity (100%) was 604.1 ± 122.3, 630.1 ± 68.6, and 937.0 ± 209.9 nmol P_i min^{-1} (mg protein)$^{-1}$ for PMV from fresh, cut and aged tissues respectively. Data are means $\pm$ S.E. of 4 or 8 independent experiments.

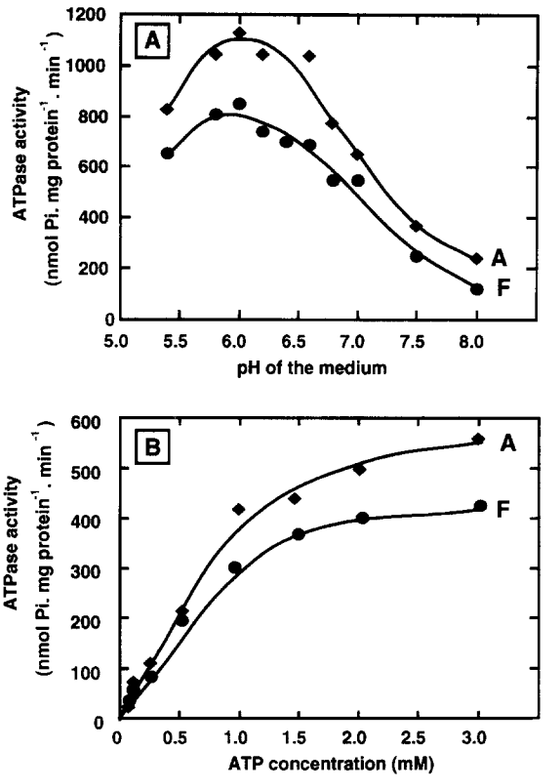

Fig. 2. pH sensitivity (A) and kinetics (B) of the vanadate-sensitive activity of PMV purified from either fresh (F) or aged (A) leaves. In A, the pH of the medium was adjusted with 50 mM Tris/Mes. In B, the kinetics were run at pH 6.5. ATPase activity was measured in the presence of 0.1 mM Na molybdate, 0.05 mM Na nitrate, and 1 mM Na azide. Data are representative results from experiments repeated four times with different samples.

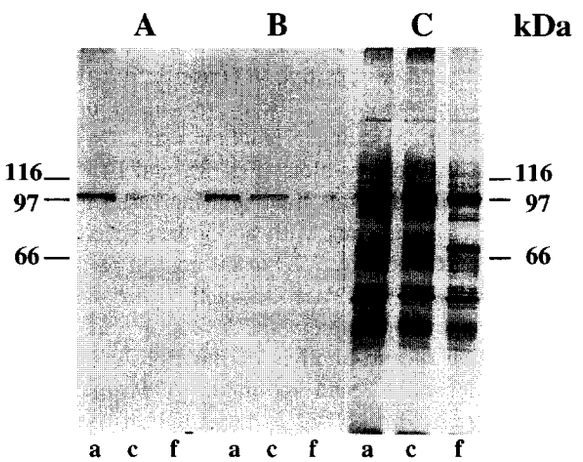

Fig. 3. Silver staining (C) and Western analysis of PM proteins from fresh, cut or aged leaves with anti-PM H^+-ATPase antibody 758 (A) and 759 (B). For Western blots, 75 μg protein were deposited in each lane; for the silver-staining, 8 μg of the corresponding samples were deposited in each lane. Position of the molecular mass markers is shown on each side. PM from fresh (f), cut (c) and aged (a) tissues. The experiments was repeated three times with independent samples and similar results.

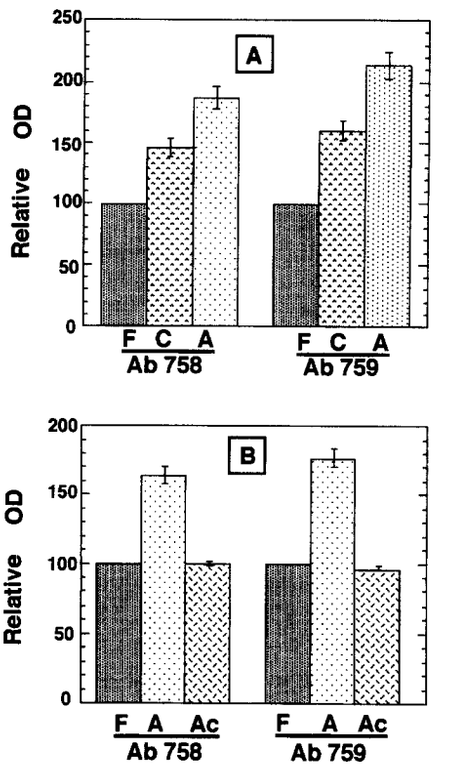

Fig. 4. ELISA estimation of the amount of PM H^+ ATPase into PMV. In A, the assays were run with PMV from fresh (F), cut (C) or aged (A) leaves, using either antibody 758 (left) or antibody 759 (right). The PMV were extracted with urea before adsorption on the immunoplates. Data are means of three measurements $\pm$S.E., taking the OD measured with fresh PMV (0.45 ± 0.10 for antibody 758; 0.325 ± 0.07 for antibody 759) as 100%. In B, the assays were run with fresh leaves (F), and with leaves aged either in the absence (A) or in the presence (Ac) of 0.1 mM CHM. The PMV were solubilized by 0.1% SDS before adsorption on the immunoplates. Data are means of two measurements $\pm$S.E., taking the OD measured with fresh PMV (0.80 ± 0.07 for antibody 758; 0.52 ± 0.10 for antibody 759) as 100%.

Western blotting of PMV from sugar beet leaves with antibody 758 (Fig. 3A) and 759 (Fig. 3B) directed against the *Arabidopsis* PM H^+-ATPase yielded a single band at 100 kDa in samples from fresh, cut and aged tissues. The position of this band is in good agreement with the molecular mass expected from the plant PM H^+-ATPases cloned sofar (104 kDa, [26]). Although silver staining of SDS-PAGE gels prepared with the different samples showed that equal amounts of proteins had been loaded (Fig. 3C), the immunoreaction was stronger with PMV from cut tissues, compared to fresh tissue, and even stronger with PMV from aged tissue (Fig. 3A and B).

Estimation of the amounts of PM H^+-ATPase were made by ELISA, using PMV purified from fresh, cut and aged tissue as the antigen, and antibodies 758 and 759 as the primary antibodies. Fig. 4 summarizes the results of these experiments. In Fig. 4A, the assays were made with PMV solubilized by urea according to O'Farrell [18], while in Fig. 4B, the PMV were only solubilized by SDS. Both ageing and cutting significantly increased the amount of

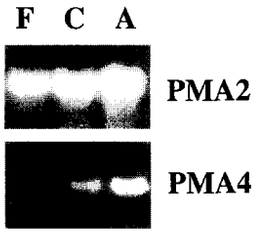

Fig. 5. Northern analysis of RNA extracted from fresh (F), cut (C) or aged (A) leaves, using the PM-ATPase probe pma2 (A), or pma4 (B). The experiments were repeated with three different extracts of each treatment with similar results. The radioactivity appears in white.

PM H^+-ATPase detected by antibody 758, but the effects of cutting were smaller than that of ageing. Similar results were obtained with antibody 759. Addition of cycloheximide during ageing prevented the increase in the amount of immunoreactive material (Fig. 4B).

The amount of total RNA recovered from fresh, cut and aged tissues were 427 ± 16, 516 ± 31, 396 ± 41 $\mu g\ g^{-1}$ fresh weight respectively (means of three extractions $\pm$ S.E.). Cutting therefore seems to increase slightly the amount of RNA in the leaf, whereas ageing had no significant effect within 12 h. Northern blots showed that the pma2 probe (Fig. 5A) and the pma4 probe (Fig. 5B) recognized a single band around 3.5 kb. Compared to fresh leaves, the level of the pma2 (Fig. 5A) transcript was increased in cut leaves, and even more in aged leaves; the level of pma4 transcript was also increased by cutting and ageing (Fig. 5B), but to a lesser extent than was the pma2 transcript. These variations were found consistently with three different RNA samples, so that they cannot be ascribed to different amounts of RNA deposited in the lanes. They are specific for the ATPase, because other probes corresponding to sugar and amino acid transporters of the plasma membrane were tested, and the transcripts did not exhibit the same changes as those found for pma2 and pma4 (S. Sakr, M. Noubahni, J. Riesmeier, N. Sauer and S. Delrot, in preparation).

4. Discussion

The PM H^+-ATPase, which plays a major role in controlling the exchanges between the plant cell and its environment, has been extensively characterized at the biochemical and molecular levels [27,28]. This enzyme exists under at least four different isoforms, and this allows for differential regulation at various levels (transcriptional, post-transcriptional, translational or post-translational) [16,20,29]. Various parameters including development [30], auxin [31] and NaCl [32] may affect the transcription of the PM proton-pumping ATPase. Post-translational control may include interactions between the C-terminal part of the enzyme and its active site [24,33,34], regulation by the redox potential, and possibly phosphorylation [27].

Plants in their natural environment have to cope with abiotic (drought, salinity, etc.) and biotic (grazing, mechanical injury by animals and insects, fungal diseases, etc.) stresses. Because they are unable to escape these stresses by mobility, unlike animals, plants have developed a wide range of responses which vary in their nature and in their lag time. Cutting and ageing are useful laboratory models mimicking various aspects of the mechanical stresses and wounding that may be undergone by plants in nature. Ageing is also the standard procedure used by plant physiologists to potentiate transport measurements [35].

The effects of cutting and ageing on assimilate transport was recently investigated in detail [4]. Cutting results in a specific stimulation of sucrose uptake in the cut leaf, whereas ageing induces a general stimulation of sucrose, 3-*O*-methylglucose, and amino acid uptake. Because inclusion of sucrose into the ageing medium did not prevent the stimulation of uptake normally induced by ageing (data not shown), we assume that this increase in the uptake capacity is related to the mechanical stress, and not to changes in the nutritional status of the tissues. The effects of cutting on sugar transport, which were shown to result from a modification of the properties of the PM [4], may be mediated by a stimulation of the activity of the proton-pumping ATPase energizing the H^+-coupled transporters and/or by a stimulation of the activity of the transporters. Increased activity may result from transcriptional or post-transcriptional control of these enzymes. The present paper aimed at a better understanding of the possible regulation of the PM H^+-ATPase induced by cutting and ageing.

Although cutting increased the levels of pma2 (Fig. 5A) and pma4 (Fig. 5B) transcripts, this did not affect the acidification of the medium by leaf fragments as measured by either recording the pH of the medium (Fig. 1) or titrating the protons extruded. Likewise, cutting affected neither the vanadate-sensitive ATPase activity (Table 1) nor the proton pumping activity measured on purified PMV. However, a stronger reaction was observed after cutting both in Western blots (Fig. 3), and ELISA assays (Fig. 4), showing that this treatment increased the amount of material cross-reacting with antibodies specific for the C-terminal part (antibody 759) or the central part (antibody 758) of the PM H^+-ATPase. Altogether, the data suggest that cutting increases rapidly the levels of transcripts and the translation of sub-families of the PM ATPase. However, this does not result in higher ATPase or proton-pumping activity of the PMV, which suggests post-translational control of the enzyme.

Ageing strongly increased the levels of pma2 and pma4 transcripts (Fig. 5), and this was accompanied by an increased amount of material reacting with anti-PM H^+-ATPase antibodies in the PM (Figs. 3 and 4), by increased vanadate-sensitive activity and proton-pumping activity of

the PMV, and by a stimulation of the acidification of the medium by the leaf tissues (Fig. 1). The appearance of immunoreactive material induced by ageing was sensitive to a CHM treatment (Fig. 4). Therefore, it resulted rather from protein synthesis than from a better targeting of preexisting ATPase to the PM. Increased ATPase activity of the PMV was due to a higher V_{max} of the enzyme, while the K_m was less affected (Fig. 2B).

Higher ATPase activity detected after ageing did not result from a proteolytic activation of the ATPase, because no additional band appeared at 93 kDa after SDS-PAGE of PMV from aged leaves (Fig. 3). Likewise, the possibility of a proteolytic-like (conformational change leading to the displacement of the autoinhibitory C-terminal domain) activation of the ATPase may be discarded because no change in the pH sensitivity of the enzyme was observed after ageing (Fig. 2A).

Altogether, the data show that the plant cells are able to respond rapidly to more or less drastic mechanical treatments by increasing the activity of the PM H^+-ATPase, a key enzyme controlling in part the exchange of solutes across the PM. The extent of the response seems to be correlated with the extent of the stress since cutting induced less dramatic effects than ageing. This response involves several steps at the transcriptional and post-translational level. The strong stimulation of ATPase activity induced by ageing is in good agreement with the general stimulation of several H^+-coupled secondary transporters (sucrose, 3-*O*-methylglucose, amino acid) induced by this treatment [4]. Yet, the specific stimulation of sucrose transport induced by cutting [4] cannot be ascribed to an increased activity of the PM H^+-ATPase energizing this transport. Indeed, the stimulation induced by cutting concerns only sucrose, and not other substrates whose uptake is also proton-driven [4]. Furthermore, cutting promotes proton-pumping activity neither in leaf tissues nor in purified PMV, although the amount of PM ATPase present in the PMV is increased (the present study). The reasons why the increased incorporation of the ATPase induced by cutting is not accompanied by increased activity remain unknown. Likewise, the nature of the primary signal and of the transductional chain leading from the mechanical wounding to the synthesis and incorporation of the PM H^+-ATPase in the membrane deserve further investigation.

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