Pergamon

PII: S0031-9422(96)00457-8

Phytochemistry, Vol. 44, No. 3, pp. 371–376, 1997

0031-9422/97 $17.00 + 0.00

CYTOSOLIC CATABOLITES OF H^+-ATPASE IN SENESCING CARNATION PETALS

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(*Received in revised form* 19 *April* 1996)

Key Word Index—*Dianthus caryophyllus*; Caryophyllaceae; petals; cytosol; H^+-ATPase; phospholipids; senescence.

Abstract—The localisation of H^+-ATPase catabolites in subcellular fractions isolated from carnation petals has been determined immunologically using two antibodies, one raised against a mid-section of the protein (amino acids 340–650) and the other corresponding to the *C*-terminus. The native M_r 100 000 polypeptide of the H^+-ATPase was clearly discernible in Western blots of microsomal membranes probed with either antibody; each antibody also depicted a distinguishable pattern of lower abundance catabolites of the protein in microsomal membranes. Some of these catabolites were present in the cytosolic fraction as well, in higher abundance than in microsomal membranes. The antibodies also depicted distinguishable patterns of other catabolites of the H^+-ATPase in the cytosol that were not present in microsomal membranes and that changed with advancing senescence of the petals. Immunocytochemical localization of the H^+-ATPase and its catabolites using the antibody specific to the *C*-terminus showed staining of the cytoplasm as well as the plasma membrane. Moreover, the cytosolic H^+-ATPase catabolites contain membrane-spanning domains of the protein and coeluted with phospholipid during Sepadex G-25 gel filtration. These observations indicate collectively that catabolites of the H^+-ATPase formed within the plasma membrane are subsequently released into the cytosol, possibly in association with lipid. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

H^+-ATPase is a plasma membrane-bound enzyme that mediates the extrusion of protons from the cytosol to the plant cell wall using ATP [1]. This ability to transform the chemical energy from ATP hydrolysis into an electrical potential difference across the plasma membrane energises key cellular functions, such as nutrient uptake [2] and cell elongation [3]. Various studies have demonstrated that the activity of H^+-ATPase is sensitive to its lipid environment. For example, phosphatidylcholine and phosphatidylethanolamine have been shown to stimulate the activity of delipidated oat root H^+-ATPase to a greater degree than phosphatidylinositol and phosphatidylserine [4]. It is also well established that H^+-pumping and ATPase activity are both dependent upon the polar head group, the length of the acyl chain and the degree of fatty acid saturation of phospholipids associated with the protein [5–9]. Indeed, interaction of H^+-ATPase with acyl chains and sterols determines its conformation, particularly that of the hydrophobic pocket [10]; perturbation of this association results in loss of enzymatic activity. For example, maize roots in cold storage experience a decline in proton-pumping activity that is attributable to loss of lipids, rather than actual degradation of H^+-ATPase [11]. The catalytic subunit of the enzyme has been identified by SDS–PAGE following phosphorylation with [^{32}P]-ATP and has a M_r of *ca* 100 000 [12]. The H^+-ATPase gene has also been cloned and sequenced, and this has led to the formulation of a model for the tertiary arrangement of the protein in the plasma membrane [13, 14]. Based on inferences from a deduced amino acid sequence, the catalytic subunit is thought to traverse the plasma membrane eight times and to have large enzymatic domains as well as both the *N*- and *C*-termini exposed to the cytosol [15].

The enzyme is known to be labile and is sensitive to degradation during isolation. Based on studies using cycloheximide to inhibit the incorporation of new H^+-ATPase into the plasma membrane, it has been estimated that the half-life of the enzyme is ~12 min [16]. Also, the Na^+-K^+-ATPase and the H^+-ATPase are both P-type ATPases; the former has been shown to be irreversibly inactivated upon exposure to activated oxygen [17]. The oxidant-modified ATPase also shows

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increased susceptibility to proteolytic attack and catabolites of the protein are detectable by SDS–PAGE following exposure to oxygen radicals [17].

Immunocytochemical techniques have been employed to identify the H^+-ATPase on the plasma membrane in several plant tissues [18–23]. However, the manner in which catabolites of the H^+-ATPase are removed from the plasma membrane has not been established. In the present study, we report the localization of H^+-ATPase catabolites in cytosol isolated from both young and senescing petals of carnation flowers. Distinguishable catabolites of the enzyme appear in the cytosol as the petals age, suggesting that there are senescence-related changes in the catabolism of H^+-ATPase.

RESULTS AND DISCUSSION

A polyclonal antibody raised against a central region of the H^+-ATPase (AA 340–650) and a monoclonal antibody raised against the *C*-terminus (AA 859–959) were used to identify catabolites of this protein in microsomal membrane preparations, which contain vesicles of plasma membrane, and in cytosol from the petals of carnation flowers. The cytosol was first subjected to ultrafiltration in order to obtain a fraction enriched in molecular complexes. The M_r 100 000 native H^+-ATPase polypeptide was clearly discernible in Western blots of microsomal membranes from both young stage 2 and senescing stage 4 petals probed with the AA 340–650 antibody (Fig. 1(A)). Low abundance catabolites of the protein were also detectable in the microsomal membrane blots probed with this antibody. Specifically, M_r 37 000, 50 000, 52 000 and 75 000 catabolites were discernible for microsomes from both ages of petal tissue (Fig. 1(A)). Of particular interest is the finding that the M_r 50 000 and 52 000 catabolites were enriched in the retentate obtained after ultrafiltration of the cytosol from stage 2 and stage 4 petals (Fig. 1(B)), but were almost undetectable in the corresponding cytosol filtrate (Fig. 1(C)). Catabolites of M_r 45 000 and 90 000, which were not detectable in corresponding microsomal membranes, were also discernible in blots of the cytosol retentate for both young stage 2 and senescing stage 4 petals (Fig. 1(A) and (B)); additional catabolites of M_r 40 000 and 80 000 were present in the cytosol retentate from older petals (Fig. 1(B)).

Western blots of microsomal membranes for both ages of tissue that were probed with the *C*-terminus antibody also featured a very intense band corresponding to the native M_r 100 000 H^+-ATPase polypeptide (Fig. 2(A)). There was also a M_r 35 000 catabolite of the protein in the microsomal blots probed with the *C*-terminus antibody that was not discernible in corresponding blots probed with the AA 340–650 antibody (Figs 1(A) and 2(A)). Different catabolites were also detectable in the cytosol with the *C*-terminus antibody. Specifically, a M_r 42 000 catabolite was clearly discernible in the cytosol retentate for young stage 2 petal tissue, and a M_r 26 000 catabolite was present in the cytosol retentate for senescing stage 4 petal tissue. These observations suggest that different catabolites of the H^+-ATPase are formed as the petals senesce. Neither of these catabolites was detectable in corresponding microsomal membranes (Fig. 2(A)), although the M_r 42 000 catabolite was present in the cytosol filtrate for both ages of tissue (Fig. 2(C)).

To determine whether there was also membrane lipid in the cytosol of carnation petals, the post-microsomal supernatant was made 10% with sucrose to increase its buoyant density, cleared of triacylglycerols by flotation centrifugation and then fractionated on Sephadex G-25. Lipid extracts of the void volume were fractionated by TLC and found to contain both esterified (phospholipid and diacylglycerol) and free fatty acids (Table 1). The lipid in the G-25 void volume appears to have been derived from membrane in that it contained the same esterified and free fatty acids as microsomal membranes, although in different relative proportions. In particular, linoleic (18:2) and linolenic (18:3) acids accounted for only 26% of the esterified fatty acid complement in the cytosolic complexes, whereas in corresponding microsomal membranes these polyun-

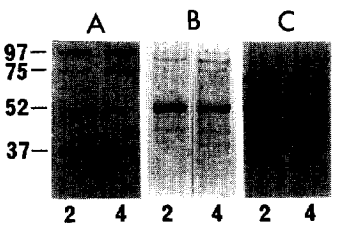

Fig. 1. Western blot analysis of subcellular fractions isolated from young stage 2 and senescing stage 4 carnation petals using an antibody raised against a specific central domain (AA 340–650) of the H^+-ATPase. The stage of petal development is indicated at the bottom of each lane. Each lane was loaded with 5 μg of protein. A, Microsomal membranes; B, retentate obtained by ultrafiltration of the cytosol; C, filtrate obtained by ultrafiltration of the cytosol. M_r markers (10^{-3}) are indicated.

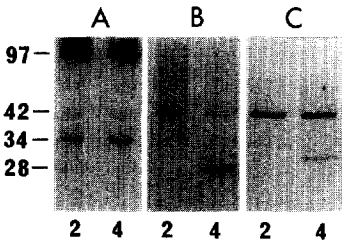

Fig. 2. Western blot analysis of subcellular fractions isolated from young stage 2 and senescing stage 4 carnation petals using an antibody raised against the *C*-terminus (AA 859–959) of the H^+-ATPase. The stage of petal development is indicated at the bottom of each lane. Each lane was loaded with 5 μg of protein. A, Microsomal membranes; B, retentate obtained by ultrafiltration of the cytosol; C, filtrate obtained after ultrafiltration of the cytosol. M_r markers (10^{-3}) are indicated.

Table 1. Fatty acid composition of microsomal membranes from stage 2 carnation petals and of lipid in the void volume obtained by Sephadex G-25 gel filtration of corresponding cytosol

Fatty acid	Microsomal membranes		Cytosol void volume	
	EST*	FFA†	EST*	FFA†
16:0	28.03±1.54	10.41±1.15	34.11±3.92	40.47±3.54
16:1	Nil	Nil	6.16±1.70	6.22±0.63
18:0	1.56±0.20	3.04±2.74	19.10±2.45	21.08±2.13
18:1	2.06±0.50	26.15±1.46	13.08±1.34	19.18±2.19
18:2	59.67±1.65	53.03±4.92	22.09±4.09	12.72±2.15
18:3	8.64±1.01	7.36±0.57	4.64±1.78	0.35±0.50

Values are means±S.E. for $n = 3$. The cytosol was cleared of triglyceride by flotation centrifugation prior to Sephadex G-25 gel filtration.

*EST, esterified (phospholipid and diacylglycerol) fatty acids.

†FFA, free fatty acids; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid.

saturated fatty acids constituted 68% of the total esterified fatty acid complement (Table 1). The free:esterified fatty acid ratio for the cytosolic lipid complement was 1.19±0.35 (S.E. for $n = 3$) compared to only 0.11±0.01 (S.E. for $n = 3$) for corresponding microsomal membranes. This indicates that the phospholipid in the Sephadex G-25 void volume was not attributable to residual small vesicles of membrane that had not been pelleted. The fatty acid compositions of the eluted fractions of cytosolic lipid were essentially similar (Fig. 3) and the lipid also co-eluted with protein and with H^+-ATPase catabolites during Sephadex G-25 chromatography (Fig. 4(A) and (B)). This may indicate an association of the cytosolic H^+-ATPase catabolites with lipid, although the prospect that separate lipid and protein complexes co-eluted in the void volume is not precluded.

Fluorescence microscopy of thin sections of petal tissue that had been treated with the monoclonal antibody specific to the *C*-terminus of the H^+-ATPase and fluorescein isothiocyanate-conjugated secondary antibody indicated that antigens recognized by the H^+-ATPase antibody are also present in the cytoplasm of intact petal cells. The plasma membranes of epidermal cells and of spongy and palisade mesophyll cells were all fluorescently labelled, which is consistent with the established subcellular localization of this enzyme; however, there was also labelling of the cell cytoplasm in these cells (Fig. 5(A), (C) and (E)). Sections incubated with pre-immunate serum showed only autofluorescence of the cell walls and there was no fluorescence in the cell cytoplasm (Fig. 5(B), (D) and (F)). Examination of the sections under visible light confirmed the highly vacuolate nature of epidermal cells (data not shown). Also, the epidermal cells fluoresced more intensely than the adjacent mesophyll cells which, as noted previously [21, 22], suggests that the level of H^+-ATPase is higher in epidermal cells than in mesophyll cells.

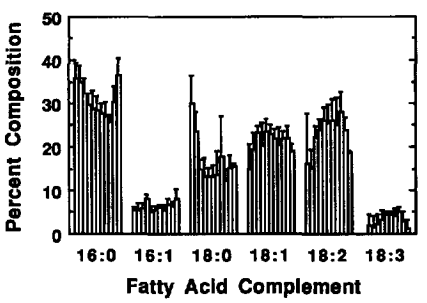

Fig. 3. Fatty acid composition of lipid fractions obtained by Sephadex G-25 gel filtration of cytosol from stage 2 carnation petals. The bars correspond to fractions 5–15 (see Fig. 4) eluted from the column. Standard errors of the means are shown for $n = 3$.

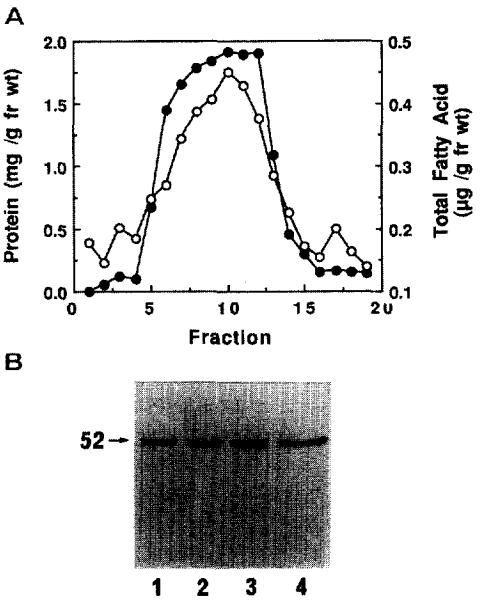

Fig. 4. Sephadex G-25 gel filtration of cytosol from stage 2 carnation petals. A, Protein (○) and total fatty acid (●) elution profiles. B, Western blot analysis using an antibody raised against the central domain (AA 340–650) of the H^+-ATPase. Lane 1, fractions 5 and 6 eluted from the column; lane 2, fractions 7 to 9; lane 3, fractions 10 to 12; and lane 4, fractions 13 to 15. The lanes were loaded with equal volume. M_r markers (10^{-3}) are indicated.

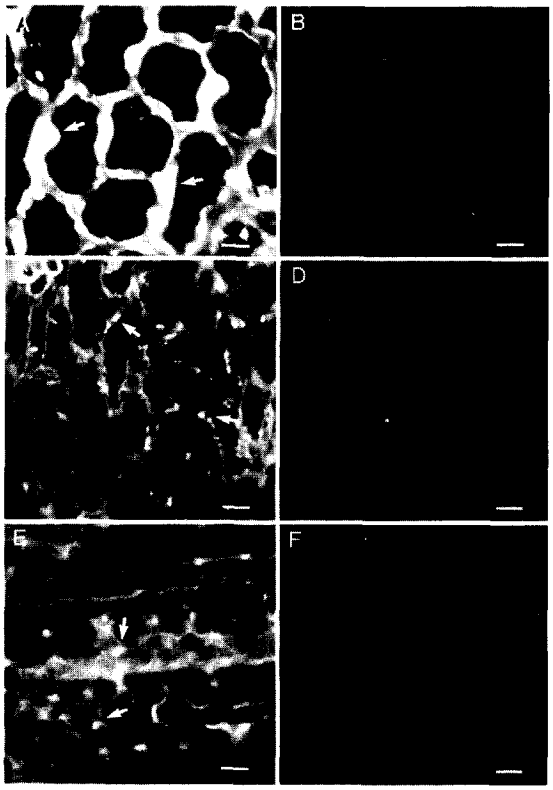

Fig. 5. Immunocytolocalisation of H^+-ATPase and its catabolites in thin-sections of stage 2 carnation petals. A, Epidermal cells incubated with the monoclonal antibody to the *C*-terminus of the protein; B, epidermal cells incubated with pre-immune serum; C, spongy mesophyll cells incubated with the monoclonal antibody to the *C*-terminus of the protein; D, spongy mesophyll cells incubated with pre-immune serum; E, palisade mesophyll cells incubated with the monoclonal antibody to the *C*-terminus of the protein; F, palisade mesophyll cells incubated with pre-immune serum. All sections were stained with fluorescein isothiocyanate-conjugated goat antirabbit IgG secondary antibody. Arrows denote antibody labelling in the cytosol. Bars = 10 μm.

Detection of the native M_r 100 000 polypeptide of the H^+-ATPase in Western blots of microsomal membrane preparations from carnation petals reflects the fact that the plasma membrane forms small vesicles during tissue homogenization that sediment as microsomes. Catabolites of the H^+-ATPase were also detectable in Western blots of microsomal membranes from both young and senescing petal tissue. This has been noted previously in isolated preparations of plasma membrane and during purification of the H^+-ATPase [22, 24–27]. The finding that these same catabolites, together with other catabolites that were not detectable in corresponding microsomal membrane preparations, were also discernible in the cytosol indicates that catabolites of the H^+-ATPase formed in the plasma membrane are subsequently released into the cytoplasm. Proteolysis was minimized by the inclusion of protease inhibitors in the homogenization buffer and in the SDS solubilizing buffer. This does not completely eliminate the possibility of catabolite formation during isolation. However, it is clear that the H^+-ATPase catabolites detectable in the cytosol are not simply hydrophylic regions of the protein that were clipped off the membrane by proteolytic cleavage during homogenization and fractionation. Specifically, the largest continuous segment of the protein protruding into the cytoplasm is a central domain comprising *ca* 360 amino acid residues with an estimated M_r of 37 000 [28], whereas the major catabolites of the H^+-ATPase detectable in the cytosol in the present study are M_r 42 000, 50 000 and 52 000 in size. This indicates that the cytosolic catabolites contain hydrophobic membrane-spanning domains of the H^+-ATPase and have been released from the interior of the plasma membrane bilayer, rather than being cleaved from its cytoplasmic surface. In addition, the M_r 50 000 and 52 000 catabolites present in the cytosol are also detectable at a much lower concentration in the microsomal membrane fraction, which supports the contention that they are formed within the plasma membrane and released after the proteolytic event. These observations collectively indicate that there is a mechanism for releasing catabolites of the H^+-ATPase into the cytoplasm after their formation within the plasma membrane. Inasmuch as this protein has a short half-life (~12 min [16]), this may well be an essential feature of H^+-ATPase turnover that precludes accumulation of its catabolites in the plasma membrane and ensuing destabilization of the bilayer structure. Monoclonal antibodies against the *C*-terminus of the H^+-ATPase recognized antigens in the cytoplasm as well as on the plasma membrane of intact petal cells. The cytoplasmic antigens presumably comprise catabolites of the H^+-ATPase, as well as newly synthesized protein. The native protein was not detectable in Western blots of isolated cytosol but newly synthesized H^+-ATPase is associated with microvesicles that would be sedimented during the high-speed centrifugation used to isolate the cytosol.

The observation that catabolites of the H^+-ATPase were enriched in the retentate obtained after ultrafiltration of the cytosol through a M_r 1 000 000 cut-off filter suggests that they may be released as a molecular complex, possibly in association with lipid because they co-elute with phospholipid during Sephadex G-25 chromatography of the cytosol. It is apparent, however, that the H^+-ATPase catabolites and phospholipid eluting in the Sephadex G-25 void volume fraction of the cytosol are not part of residual microsomal membranes, since the void volume constituents exhibit a much higher free to esterified fatty acid ratio than is characteristic of membranes. Also, the native M_r 100 000 H^+-ATPase polypeptide, which was prominently featured in Western blots of microsomal membranes, was not detectable in corresponding Western blots of Sephadex G-25-purified cytosol protein, whereas catabolites of the protein were discernible.

In summary, our observations indicate that once catabolites of H^+-ATPase are formed in the plasma

membrane, they are released into the cytosol, possibly as a molecular complex. This may be a means of preventing the accumulation of protein catabolites in the plasma membrane, which might otherwise destabilize its structure.

EXPERIMENTAL

Plant material and subcellular fractionation. Carnation flowers (*Dianthus caryophyllus* L. cv. White Sim) were obtained from a commercial grower. Flowers were harvested at an early stage when the petals had extended *ca* 2 cm beyond the sepals. They were maintained in deionised H_2O at 22° until specific stages of post-harvest development had been reached, namely stage 2 in which the petals were fully expanded and the flowers fully open with yellow tinted centres, and stage 4 by which time the petals were showing pronounced inrolling and had begun to senesce [29].

Petals were homogenized (25 g fr. wt 150 ml^{-1}) at 4° in isolation buffer (50 mM Epps–0.25 M sorbitol pH 7.4, 10 mM EDTA, 2 mM EGTA, 1 mM PMSF, 1 mM benzamidine and 20 μg/ml chymostatin) for 45 sec in an Omnimixer and for an additional min in a Polytron homogenizer. The homogenate was filtered through four layers of cheese-cloth and the filtrate centrifuged at 10 000 *g* for 20 min at 4°. The pellet was discarded and microsomal membranes were isolated by centrifugation of the supernatant for 1 hr at 250 000 *g*. Microsomal membranes were washed by resuspension in 5 vols of isolation buffer containing 50 $\mu g\ ml^{-1}$ chymostatin and centrifugation at 250 000 *g* for 1 hr and then resuspended in isolation buffer containing 50 $\mu g\ ml^{-1}$ chymostatin at a concentration of 2 mg protein ml^{-1}.

The cytosol (post-microsomal supernatant) was centrifuged at 305 000 *g* for 12 hr in order to sediment any residual membrane and then passed through a M_r 1 000 000 cut-off filter (Pharmacia) in order to obtain a fr. (the retentate) enriched in molecular complexes. For this purpose, 150 ml of cytosol was reduced to 10 ml and the 10 ml retentate was then washed 3× with 3 vols of isolation buffer using the same filter and reduced to a final retentate vol. of 10 ml. In other expts, the cytosol was made 10% (w/w) with sucrose and subjected to flotation centrifugation in order to remove triacylglycerols. Each 23 ml of sucrose-adjusted cytosol was overlaid with 1.5 ml of homogenization buffer and centrifuged at 305 000 *g* for 12 hr. Triacylglycerols floated into the upper layer and the triacylglycerol-cleared cytosol was then used directly for analysis or fractionated further by Sephadex G-25 gel filtration. For this purpose, 3 ml of cytosol followed by 8 ml of homogenizing buffer were applied to a column containing 8.5 ml (bed vol.) of Sephadex; 1 ml fs were collected by gravity filtration.

Analytical procedures. Total lipids were extracted from microsomal membranes and fractionated cytosol according to ref. [30] and separated by TLC [31]. The separated lipids were detected with I_2 vapour and identified using authentic standards. Free fatty acids and esterified (phospholipid and diacylglycerol) fatty acids were eluted from the silica gel, methylated according to ref. [32] and analysed by GC [33]. In some expts, total lipid extracts were methylated directly for fatty acid analysis without further fractionation.

Protein was quantified according to ref. [34] using BSA (Sigma) as standard. Polypeptides were fractionated by SDS–PAGE in Mini Protean Dual Slab Cells (Bio-Rad) using 12% acrylamide. For this purpose, samples were dissolved in SDS buffer containing 2 mM PMSF and 100 $\mu g\ ml^{-1}$ chymostatin [35]. H^+-ATPase and its catabolites were localized by Western blotting and immunodetection using a polyclonal antibody raised against a central domain (AA 340–650) of the H^+-ATPase [35] and a monoclonal antibody raised against the *C*-terminus (AA 859–959) of the protein (gift from M. R. Sussman).

Immunocytochemistry. Fixation and dehydration were performed essentially according to ref. [36]. Petals of stage 2 flowers were cut into small pieces (2 mm^2) and immediately fixed with 2% paraformaldehyde–2% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2, containing 0.15 M sorbitol and 1 mM $CaCl_2$ for 5 hr. Petal pieces were vacuum infiltrated for 20 min at the start of fixation. Samples were subsequently washed with Na-cacodylate buffer and taken to dist. H_2O over a 12 hr period and then dehydrated to 90% EtOH. Following dehydration, petal pieces were embedded in LR White resin and 1–2 μm sections cut with a glass knife.

Tissue sections were transferred to glass slides, overlaid with 50 mM NH_4Cl in TBS (20 mM Tris–HCl, 150 mM NaCl, pH 8.0) for 10 min to block free aldehydes and treated with antibodies essentially as described in ref. [37]. TBS was supplemented with 0.05% (v/v) Tween 20 (TBST) and 1% (w/v) BSA for the antibody-incubation steps and with 0.5% (v/v) Tween 20 (TBST) and 1% (w/v) BSA for the washes. Following a 30 min incubation with TBST plus 1% BSA, sections were overlaid with primary antibody (monoclonal antibody specific to the *C*-terminus of the H^+-ATPase) or preimmune serum diluted 1:500 for 3 hr. After 3 washes (10 min each) with TBST plus 1% BSA, sections were overlaid with fluorescein isothiocyanate-conjugated goat antirabbit IgG (Sigma) for 4 hr in the dark. Sections were then washed 3× (10 min each) with TBST plus 1% BSA and once (10 min) in TBS. Slides were mounted in glycerol:TBS (4:1), viewed under a Zeiss Axiphot Photomicroscope III equipped for epifluorescence and photographed on Kodak ektachrome 400 ASA film.

Acknowledgements—The authors are grateful to R. Serrano, European Molecular Biology Laboratory, Heidelberg, Germany, for providing the antibody against the central domain (AA 360–650) of the H^+-ATPase, and to M. R. Sussman, Department of Horticulture, University of Wisconsin, Madison, Wisconsin,

U.S.A., for providing the antibody against the *C*-terminus of the H^+-ATPase. Financial support in the form of a grant-in-aid from the Natural Sciences and Engineering Research Council of Canada is also gratefully acknowledged.

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