Planta (1995) 196:15–22

Planta

Discrete subcellular localization of membrane-bound ATPase activity in marine angiosperms and marine algae

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Received: 21 June 1994 / Accepted: 14 September 1994

Abstract. The subcellular distribution of membrane-bound ATPases was compared among terrestrial plants, seagrasses and marine algae by cytochemical techniques. High ATPase activity was detected in the copiously invaginated plasma membrane that was characteristic of transfer cells but not in the tonoplast of epidermal cells in mature leaves of seagrasses. Magnesium- or Ca^{2+}-dependent ATPase activity was induced together with the characteristics of transfer cells during the development of leaf tissues able to resist seawater. Northern hybridization revealed the effective induction of the synthesis of mRNA for plasma-membrane H^+-ATPase during the development of leaves. Such high ATPase activity was not detected in the smooth plasma membranes of marine macro-algae but was found in the membranes of some cytoplasmic vesicles or microvacuoles, providing evidence of the excretion of salts by exocytosis. It appears, therefore, that two essentially different methods for excreting excess salts have developed separately in these two classes of marine plants. The evolution of mechanisms of salt tolerance in the plant kingdom is discussed in terms of the differential subcellular distribution of ATPase activity.

Key words: H^+-ATPase – Marine alga – Ouabain sensitivity – Seagrass – Seawater tolerance – Transfer cell

Introduction

The ability to tolerate high salinity is one of the most important examples of genetic adaptation in plants. Terrestrial plants have almost entirely lost their tolerance to high salinity and/or their ability to thrive in seawater during the course of their evolution. However, tolerance mechanisms have evolved anew in some angiosperms, namely seagrasses, and allow such plants to thrive in seawater. Thus, the ability to resist seawater has developed phylogenetically in the plant kingdom, a phenomenon that suggests the presence of highly genetically controlled tolerance mechanisms in these marine plants. It is clearly important to determine whether essential differences exist between the salt-tolerance mechanisms of the two evolutionarily distinct classes of marine algae and marine angiosperms. In addition to advancing studies on various terrestrial halophytes, comparison of the salinity-tolerance mechanisms of these two classes of marine plants may be relevant to attempts at the genetic improvement of resistance in salt-sensitive crops.

Salt-transport systems involving ATPases that are located in cellular membranes are important for a description at the molecular level of the salinity tolerance of plants. It is widely accepted that the various types of ion-pumping ATPase are well conserved among a variety of plants and that tonoplast- and plasma-membrane-bound ATPases have distinct structures (for reviews, see Sze 1985; Nelson 1988). In general, it appears that the former ATPases play more important roles than the latter ones in the restricted salt-tolerance ability of terrestrial plants; when some higher plants are subjected to salt stress, ATPases are often activated in the tonoplast and excess salt is accumulated in the large vacuoles, while the plasma-membrane-bound ATPase activity sometimes decreases (Erdei et al. 1980; Walker and Leigh 1981; Blumwald and Poole 1985; Garbarino and Dupont 1988; Sanchez-Aguayo et al. 1991). However, it has been found, especially in some halophytes, that salt induces enhanced H^+-translocation, ATPase activity of the plasma-membrane H^+-pump and gene expression of the ATPases (Braun et al. 1986a, b; Niu et al. 1993a). On the other hand, no reports on the subcellular distribution of ATPase activity have been presented for either of these two classes of marine plants, although some ATPases in membrane preparations from several marine algae have been partially characterized (Graves and Gutknecht 1977; Okazaki 1977; Wada et al. 1989; Ikeda et al. 1990).

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Abbreviation: PCR = polymerase chain reaction

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We have found that, in seagrasses, cells isolated from meristematic and immature-leaf tissues that are protected from seawater by tightly enveloping sheaths are more sensitive to salinity, while mature-leaf cells with the morphological characteristics of transfer cells (Jagels 1973, 1983; Birch 1974; Doohan and Newcomb 1976; Barnabas et al. 1977, 1982; Kuo et al. 1988, 1990) are highly resistant to a wide range of osmotic potential and salinity (Arai et al. 1991). Transfer cells of terrestrial plants are highly active in transporting salts and nutrients (for reviews, see Gunning and Pate 1969; Gunning 1977). The highly invaginated plasma membranes of the typical transfer cells are associated with high levels of ATPase activity (Maier and Maier 1972; Bentwood and Cronshaw 1978; Evert et al. 1988; Katz et al. 1988; Parets-Soler et al. 1990; DeWitt et al. 1991; Villaba et al. 1991; Wimmers and Turgeon 1991). Such high invagination of the plasma membrane increases its surface area and, thus, the number of potential pump sites in the plant cell (Wimmers and Turgeon 1991). Hence, the differentiation of transfer-cell-like structures, particularly in the seawater-resistant tissues, suggests that the plasma-membrane-bound ATPases in the epidermal cells of seagrasses are important for the plants to thrive in seawater. On the other hand, no characteristics of transfer cells have ever been observed in marine algal cells, with the exception of the epidermal cells in coralline red algae that function in the accumulation of calcium (Borowitzka and Vesk 1978; Bressan et al. 1981). These observations suggest that there is a primary difference in the mechanisms for salt tolerance of marine algae and seagrasses.

In this report, we describe the discrete subcellular localization of membrane ATPases with particular reference to seagrasses and marine algae. Evolution of salinity-resistance mechanisms is discussed in terms of the subcellular distribution of membrane-bound ATPases in these classes of marine plants and in land plants.

Materials and methods

Materials. *Zostera marina* L. and *Z. japonica* Ascherson were collected at Futtsu in Chiba Prefecture. *Bryopsis* sp., *B. maxima* Okamura, *Ulva arasakii* Chihara, *U. pertusa* Kjellman, *Chaetomorpha crassa* (Agardh) Kuetzing, *Cladophora opaca* Sakai, *Grateloupia filicina* (Wulfen) C. Agardh, and *Eisenia bicyclis* (Kjellman) Setchell were harvested in Tokyo Bay. Samples were kept in the light at 20° C in artificial seawater (Aqua Marine; Yashima Pure Chemical Co. Ltd., Osaka, Japan) or natural seawater. The plants were used for studies within one week of harvest. An established culture of *Porphyra tenera* Kjellman was graciously provided by Dr. S. Araki, Yamamotonori Research Laboratory, Tokyo.

Terrestrial (*Impatiens balsamina* L., *Galium aparine* L., *Statice armeria* L., *Trifolium repens* L., *Cucumis sativas* L. and *Pisum sativum* L.) and limnetic (*Vallisneria spiralis* L.) plants were cultured in a greenhouse.

Cytochemical detection of ATPases. For detection of ATPase activity, materials were fixed in 1% (v/v) formaldehyde or 2.5% (v/v) glutaraldehyde in 50 mM cacodylate buffer, for 1 h at 4° C, and then washed for 1 h at 4° C in the same buffer and for 30 min in 50 mM Tris-malate (pH 7.0). These reagents are known to inhibit the ATPase activity in the mitochondrial membrane but to preserve adequately the enzymatic activities associated with other membrane systems (Winter-Sluiter et al. 1977; Chauhan et al. 1991). The fixed and washed tissues were placed in an incubation medium with or without 10^{-1} mM sodium vanadate, 10^{-3} mM ouabain, or 50 mM KNO_3 and were incubated for 2 h at room temperature. Lead precipitation, specific for ATPase activity, was carried out as described by Hall et al. (1980). Although the validity of this procedure itself has often been disputed (Katz et al. 1988; Evert et al. 1988; Chauhan et al. 1991), recent studies by immunolocalization (Parets-Soler et al. 1990; Villaba et al. 1991) and reporter-gene expression (DeWitt et al. 1991) have shown that plasma-membrane H^+-ATPase activity is highly concentrated in vascular tissues that contain transfer cells, as suggested by the cytochemical procedure.

Some sections were post-stained with 2% (w/v) uranyl acetate for 15 min. The fixed tissues were dehydrated in a graded acetone or ethanol series, embedded in Spurr's (1969) resin mixture, and sectioned. The sections were observed under an electron microscope (JEOL 100C; Nihon Densi, Co., Tokyo, Japan).

Northern hybridization. The radiolabeled probe for hybridization was prepared from pZM7 [which contained a 430-base-pairs (bp), polymerase chain reaction (PCR)-amplified fragment of DNA] with a random-primer labeling kit (Takara, Kyoto, Japan) and α-[^{32}P]dCTP (Amersham, Arlington Heights, Ill., USA). Two oligonucleotides (5′-GAICAGTCTGCTCTIACICG-3′ and 5′-GTCC-

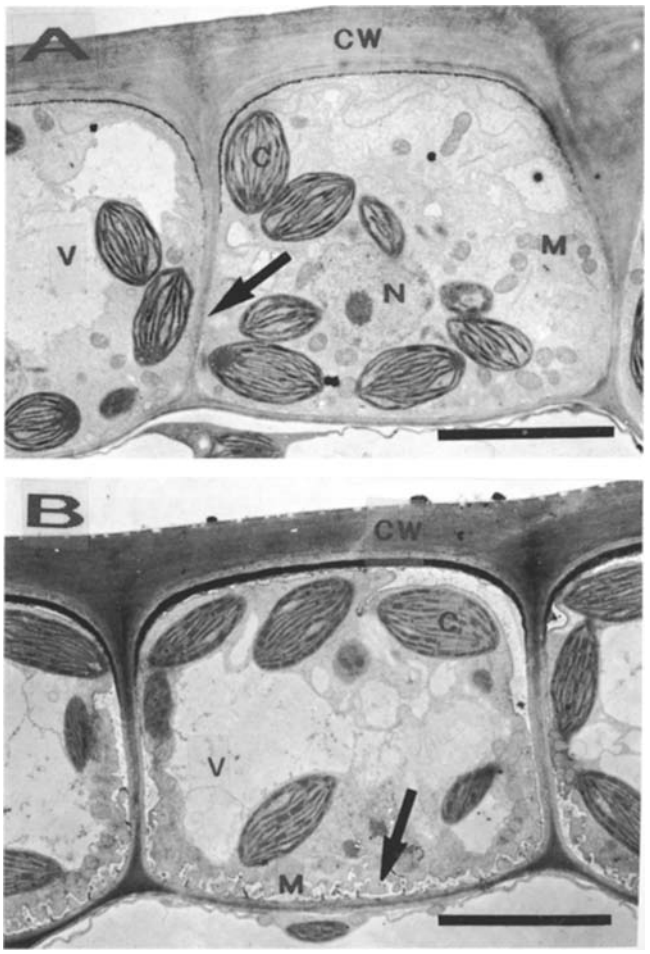

Fig. 1A, B. Induction of characteristics of transfer cells in epidermal tissues during leaf development of *Zostera marina*. **A** Immature-leaf cells; **B** mature-leaf cells. Note the smooth (**A**) and highly invaginated (**B**) plasma membranes indicated by *arrows*. *N*, nucleus; *C*, chloroplast; *M*, mitochondria; *CW*, cell wall; *V*, vacuole. ×2300; bars = 10 μm

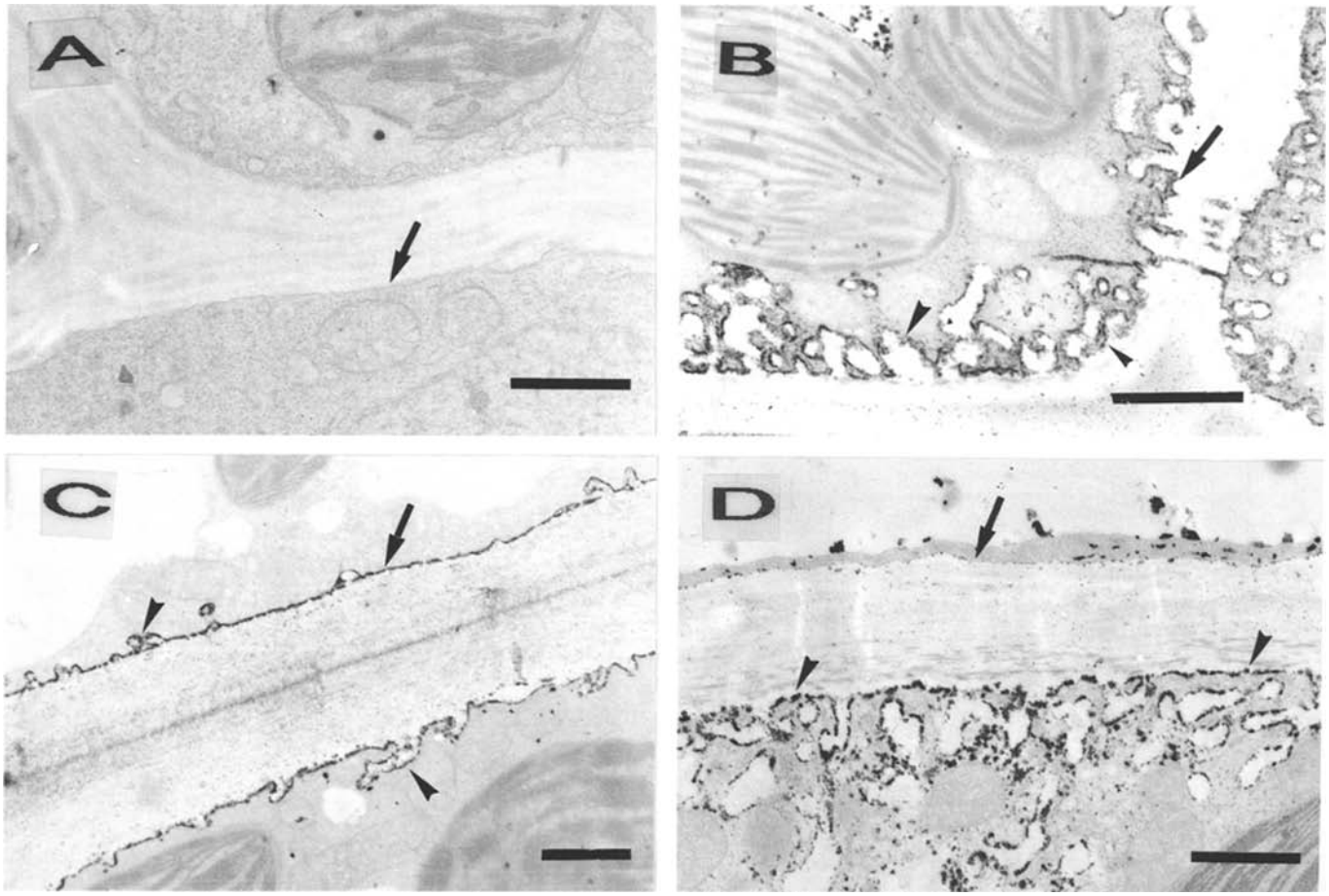

Fig. 2A–D. Distribution of ATPase activity in epidermal cells of leaves of *Z. marina*. Post-staining by uranyl acetate was omitted. Plasma membranes are indicated by *arrows* and lead precipitates by *arrowheads*. Bars = 1 μm. **A** Epidermal cells of immature leaves. ATPase activity is undetectable along the smooth plasma membranes (× 16 000). **B** Epidermal cells of mature leaves. High ATPase activity is detected along the highly invaginated plasma membranes (× 16 000). **C** Epidermal leaf cells just before release of leaves from sheaths. They are in the early stages of the induction of transfer cell-like structures. Both smooth and invaginated plasma membranes are active (× 12 000). **D** Parenchymal and epidermal cells of mature leaves. High ATPase activity is found in neither tonoplast nor plasma membrane of the upper parenchymal cell. The lower cell is epidermal (× 14 000)

CIGTITTGTCACTICA-3′), designed by reference to consensus amino-acid sequences of the plasma-membrane H^+-ATPase for higher plants (PQSALTG and CSDKTGT) (Wada et al. 1992), were used as primers for PCR. High-molecular-weight chromosomal DNA that was purified from *Z. marina* leaves by the cetyl trimethylammonium bromide method (Rogers and Bendich 1988) was used as a template for PCR amplification. The nucleotide sequence of the 430-bp fragment of DNA was determined with a Sequenase Ver. 2.0 kit (U.S.A. Biochemical Corp., Cleveland, Ohio, USA). Analysis of nucleotide sequences and the homology search was performed using SDC-GENTYX programs for processing of genetic information (Software Development Co., Tokyo, Japan).

Total RNA was isolated from 5 g of immature or mature leaves of *Z. marina* L. by the acid guanidium-phenol-chloroform method (Chomeczynski and Sacchi 1987). Twenty micrograms of isolated RNA was fractionated in a 1.2% (w/v) agarose gel that contained 3-(N-morpholino)propanesulfonic acid (Mops) buffer (pH 7.0), 0.66 M formaldehyde and 500 ng · ml^{-1} ethidium bromide. RNA was then transferred to a nylon membrane (Zeta-Probe; BioRad., Richmond, Calif., USA). Hybridization was carried out in hybridization medium (0.25 M sodium phosphate (pH 7.2), 1 mM ethylendiaminetetraacetate (EDTA), 7% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) bovine serum albumin (BSA), 1% (v/v) Nonidet P-40 at 65° C for 16 h. The membrane was washed twice with 20 mM sodium-phosphate buffer (pH 7.2) containing 5% SDS at 65° C for 30 min each, and then washed twice again with 20 mM phosphate buffer containing 1% SDS at 65° C for 30 min each.

Results

Induction of ATPase activity in epidermal cells with characteristics of transfer cells during development of seagrass leaves. The meristematic tissues of *Zostera marina, Z. japonica* and *Phyllospadix iwatensis* leaves, which are tightly protected from seawater by long leaf sheaths in their basal regions, have no invaginated cell membranes (Arai et al. 1991) (Fig. 1A). The induction of unusual cell structures in the epidermis is found in the zone about 3 cm from the basal meristematic region of leaf blades. The characteristic structures, in particular the copious invagination of the cell membrane, place the cells within the class of transfer cells of terrestrial plants (Fig. 1B). Many mitochondria are localized around the invaginated cell membrane. In leaf-epidermal cells, invaginations of

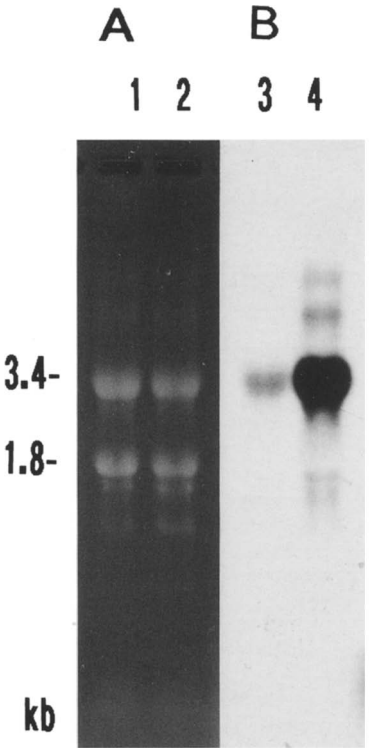

Fig. 3A, B. Expression of the putative gene(s) for a plasma-membrane H^+-ATPase in leaves of *Z. marina*. The hybridization probe was prepared from pZM7 (see text). Samples (20 μg) of total RNA isolated from immature leaves *(lanes 1, 3)* or mature leaves *(lanes 2, 4)* were analyzed. **A** Agarose-gel electrophoresis; **B** Northern hybridization

the cell membrane are less abundant on the side closest to the cuticle, while on the inner side the cell membrane is highly invaginated.

Because the level of cytochemical detection of ATPase activity by lead precipitation is not very high, enzymatic activity was almost undetectable along the smooth cell membranes of tissues of immature leaves prior to their release from the leaf sheaths (Fig. 2A). However, extremely high ATPase activity in association with the copiously invaginated cell membranes of epidermal tissues was detectable in mature leaves (Fig. 2B). The tonoplast had low levels of ATPase activity in the epidermal cells of both mature and immature leaves. These observations suggest that high ATPase activity is induced exclusively in epidermal cells as the leaves develop the ability to resist seawater. Although the activation of plasma-membrane-bound ATPase and the induction of transfer-cell-like structures appeared to be correlated during leaf development of seagrasses, these two phenomena seem to be regulated independently; both smooth and invaginated plasma membranes were associated with similarly high ATPase activity in one epidermal cell during an early stage in the induction of transfer-cell-like structures (Fig. 2C).

On the other hand, internal parenchymal tissue cells of mature leaves did not have high ATPase activity (Fig. 2D) except sometimes in the tonoplasts of their large vacuoles. Insignificant activity was detectable in the epidermal leaf cells of *Vallisneria spiralis*, a limnetic monocotyledonous plant which has thin leaf blades like seagrasses but no features characteristic of typical transfer cells (not shown).

Induction of mRNA for plasma-membrane H^+-ATPase in seagrass leaves. We constructed a probe specific for the gene for the plasma-membrane H^+-ATPase of *Z. marina* using PCR. The probe, pZM7, was designed to contain a 430-bp fragment of this gene. Homologies in terms of nucleotide and deduced amino-acid sequence between the 430-bp fragment and the corresponding region of the gene for the plasma-membrane H^+-ATPase of rice (*Oryza sativa* L.) (Wada et al. 1992) were 68.7% and 82.5%, respectively.

As shown in Fig. 3 (A, B), putative gene(s) for a plasma-membrane H^+-ATPase in mature-leaf cells was expressed at levels at least ten times higher than in immature-leaf cells. This result is consistent with the observations made by cytochemical methods (Fig. 2A, B). The length of the main mRNA detected by Northern hybridization was estimated to be about 3.4 kb by comparing its mobility with that of 25S rRNA (3.4 kb). The mRNA was similar in length to mRNAs for plasma-membrane H^+-ATPases in terrestrial higher plants (Boutry et al. 1989; Harper et al. 1989; Ewing et al. 1990; Niu et al. 1993a).

Specific inhibition of membrane ATPase activity in seagrass cells. The reaction catalyzed by ATPase in seagrass cell membranes was effectively inhibited by 10^{-1} mM sodium vanadate (Fig. 4A) as is also the case in terrestrial plants (Erdei et al. 1980; Nelson 1988). To our surprise, ouabain, which is a specific inhibitor of animal K^+/Na^+-ATPases (Jørgensen 1982), considerably reduced the extent of the cytochemical reaction in the fixed seagrass epidermis at 10^{-3} mM (Fig. 4B). The reagent did not inhibit the ATPase activity associated with the tonoplast in seagrass cells. However, the presence of K^+ or Na^+ ions in the reaction medium did not stimulate the ATPase activity of the cell membrane as monitored by the cytochemical procedure.

The ATPase activity in seagrass cell membranes was highly dependent on the presence of 1 mM Ca^{2+} or Mg^{2+} in the reaction medium at pH 7–8 (Fig. 4C, D). Cell membranes are a likely target for the toxic effects of high salinity on terrestrial plants and a protective effect of Ca^{2+} in the presence of excess Na^+ has been observed (Leopold and Willing 1984). In fact, leaf blades of seagrass became brownish in color and most of leaf cells died within several days in 0.5 M NaCl (not shown). This phenomenon could be prevented by the addition of 1 mM $CaCl_2$ to the medium. Since leaf cells of seagrass remained alive, for several days at least, in a medium that contained 0.5 M sorbitol without Ca^{2+}, the observed toxic effects on the leaf cells could be attributed to high salinity and not to a lack of Ca^{2+} in the medium. Thus, cell membranes of seagrasses appear to be essentially as sensitive to high salinity as terrestrial plant cells. However, the toxic effects of high salinity on the seagrass cells could not be protected by Mg^{2+} (not shown), which can

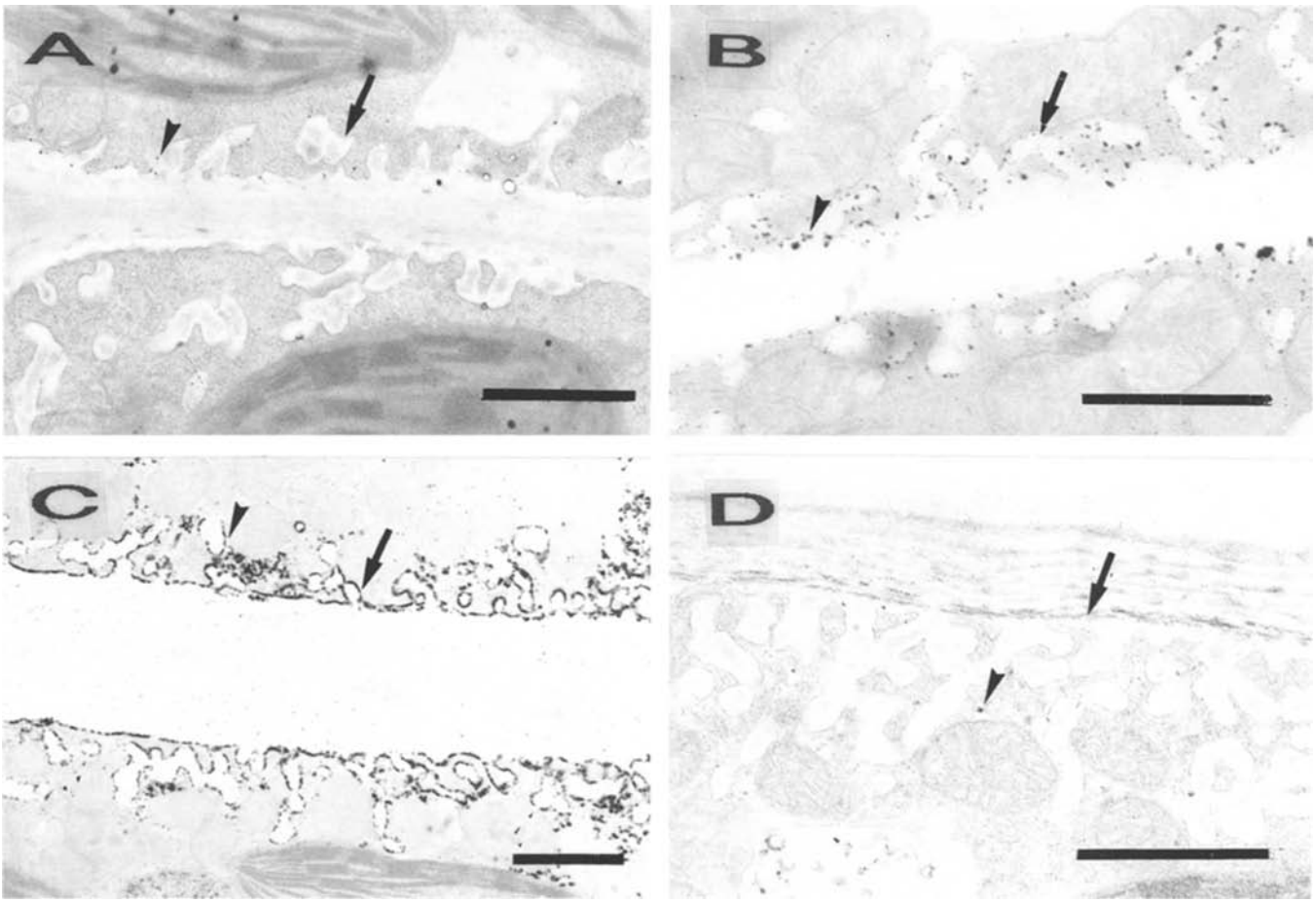

Fig. 4A–D. Inhibition of ATPase activity in plasma membranes of mature leaves of *Z. marina*. *Arrows* indicate plasma membranes and *arrowheads* lead precipitates. Post-staining was omitted. For control, see Fig. 2B. Bars = 1 μm. **A** Inhibitory effect of 10^{-1} mM sodium vanadate (×20 000). **B** Inhibitory effect of 10^{-3} mM ouabain (×25 000). **C** Substitution of Ca^{2+} for Mg^{2+} in the reaction medium (×15000) **D** Absence of divalent cations in the reaction medium (×25 000)

be completely substitute for Ca^{2+} in the reaction medium for membranous ATPases. Anyhow, Ca^{2+} in seawater very likely plays an important role in allowing seagrasses to thrive in seawater.

Differential localization of ATPases in marine algal cells and terrestrial plant cells. Unlike epidermal cells of seagrass leaves, the cells of the marine green *(Bryopsis* sp., Fig. 5A; *B. maxima; Ulva arasakii; U. pertusa,* Fig. 5B; *Chaetomorpha crassa; Cladophora opaca),* red *(Porphyra tenera,* Fig. 5C; *Grateloupia filicina)* and brown *(Eisenia bicyclis)* macro-algae that we examined had quite low levels of ATPase activity in their plasma membranes. Instead, membranes of some cytoplasmic vesicles and/or tonoplasts of microvacuoles were stained, often somewhat heterogeneously, and some vacuoles were closely associated with each other (Fig. 5A–C). The membrane-bound ATPase activity detectable in various marine algae was insensitive to sodium vanadate and ouabain but it was quite sensitive to KNO_3, which inhibits tonoplast-bound ATPases.

Such high levels of ATPase activity were never found in the epidermal leaf cells of the terrestrial plants *(Impatiens balsamina, Galium aparine, Statica armeria, Trifolium repens, Cucumis sativus* and *Pisum sativum)* that we examined by the cytochemical procedure. Enzymatic activity was also undectable in meristematic tissue cells of these plants. However, in agreement with previous reports (Bentwood and Cronshaw 1978; Hall et al. 1980; Sanchez-Aguayo et al. 1991), considerably high activity was often localized either in the plasma membrane or in the tonoplast depending on the tissues (Fig. 6A, B).

The different subcellular localization of ATPase activity in terrestrial plants, seagrasses and marine algae is related to the importance of each pump type in the primary mechanism for regulating excretion of nutrients and salts and thus, presumably, the salt tolerance of these plant cells.

Discussion

Our cytochemical observations indicate the occurrence of high ATPase activity, very likely responsible for seawater tolerance, in the plasma membrane of seagrass epidermal cells (Fig. 2). In contrast, enzymatic activity was detectable only in the tonoplast of marine algal cells (Fig. 5). The cytochemical procedure was useful for

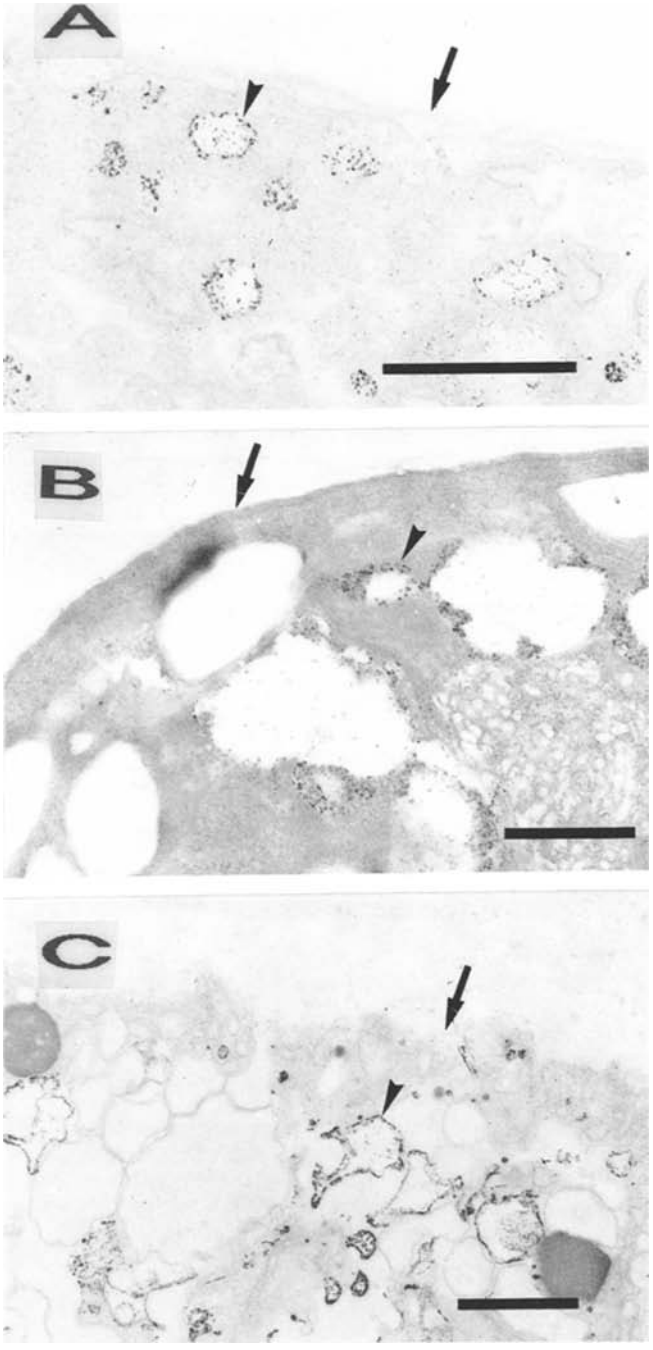

Fig. 5A–C. Distribution of ATPase activity in cells of marine algae. Post-staining was omitted. Bars = 1 μm. **A** *Bryopsis* sp. (green alga). No enzymatic activity is detectable along algal plasma membranes. Instead, various types of vesicle can be identified by lead precipitation in the algal cytoplasm (× 25 000). **B** *Ulva pertusa* (green alga) (× 16 000). **C** *Porphyra tenera* (red alga). Closely associated vacuoles are visible (× 16 000)

demonstrating the different subcellular localizations of ATPases in these plants. In harmony with the cytochemical observations, Northern hybridization clearly demonstrated high levels of mRNA for plasma membrane H^+-ATPase in mature seagrass leaves (Fig. 3).

The most significant morphological feature of seawater-resistant epidermal cells of seagrass leaves is the complex cell membrane, associated with extraordinarily high ATPase activity (Figs. 1, 2). Most of salt-gland cells of various halophytes also have such transfer-cell-like characteristics (for reviews, see Gunning and Pate 1969; Gunning 1977; Lüttge 1971). These observations suggest that transfer cells are utilized for excretion of excess salts from the epidermis of these kinds of salinity-tolerant plants and that the cells are very important if we are to understand the nature and evolution of salinity-tolerance mechanisms in plants.

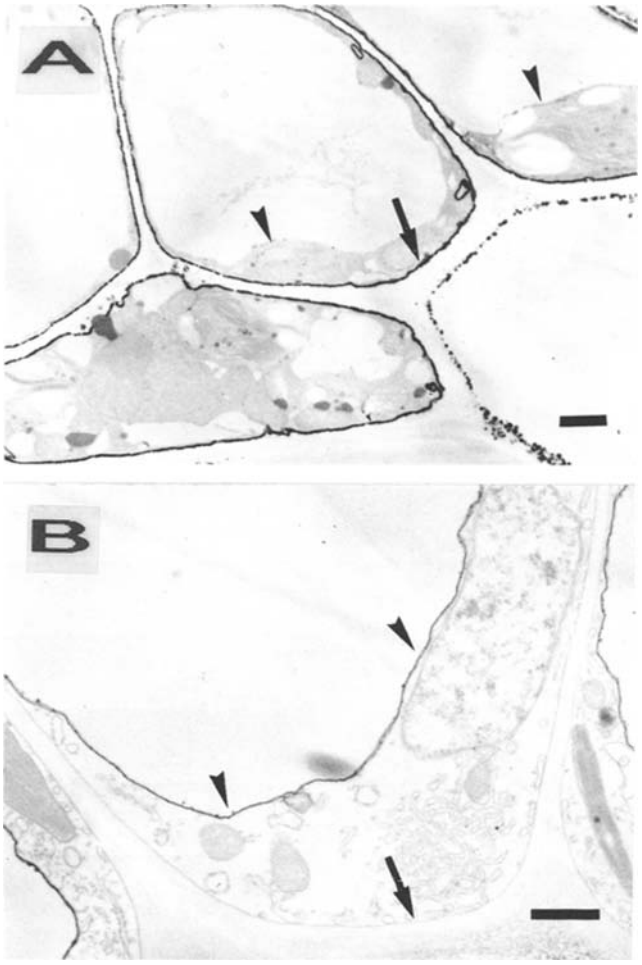

Fig. 6A, B. Differential subcellular localization of ATPase activity around vascular tissues (**A**) and parenchymal tissues (**B**) of *Galium asparine* stems. Post-staining was omitted. Plasma membranes are indicated by *arrows* and tonoplasts by *arrowheads*. ATPase activity is detectable either in plasma membranes (**A**) or in tonoplasts (**B**) in the cells of these tissues × 5000 (**A**), × 8000 (**B**); bars = 1 μm

However, the properties of transfer cells are still scarcely known because typical transfer cells, interspersed along the vascular system of terrestrial plants, are not easily accessible for experimentation. In this regard, the epidermal cells from seagrass leaves are of particular interest; these cells can be isolated in large numbers for direct studies of their properties (Arai et al. 1991). The differentiation of transfer cells in a variety of tissues and organs of terrestrial plants is genetically and physiologically regulated (Pate et al. 1970; Jones and Northcote 1972; Yeung and Peterson 1975; Kramer 1978; Kramer

et al. 1980; Folsom and Cass 1986). Under salt-stress conditions, some transfer cells are induced in the root epidermis of certain terrestrial plants (Kramer 1978; Kramer et al. 1980). Thus, we hypothesize that genetic systems for induction of specialized transfer cells are activated in developing epidermal tissues of seagrasses for development of the ability to tolerate the high salinity and osmolarity of seawater. It will be of interest to determine whether or not the induction of transfer cells in leaves of seagrass is dependent on the presence of seawater.

Transfer cells do not develop in marine macro-algae with the exception of coralline algae (Borowitzka and Vesk 1978; Bressan et al. 1981). Instead, the marine algal cells had numerous cytoplasmic microvacuoles or vesicles with membranes that had rather high ATPase activity (Fig. 5). It is probable that mechanisms for tolerance to salinity involving excretion of excess salts via exocytosis of microvacuoles have evolved effectively in marine algae.

It appears that most terrestrial plant cells develop larger vacuoles and thus lose the ability to excrete salts via the exocytotic membrane-transfer system even if the vacuoles are able to accumulate significant amounts of excess salts. On the other hand, terrestrial plants have developed characteristic vascular systems for translocation of nutrients and salts and thus, in most terrestrial plants, high levels of ATPase activity can be localized in plasma membranes of the vascular tissues and in tonoplasts of other differentiated tissues (Fig. 6).

Thus, plant cells can currently exploit these two primary methods or "strategies" for excreting excess salts and the discrete methods appear to have evolved independently in the two evolutionarily separate groups, seagrasses and marine algae for resisting the high salinity of seawater. It would be a biological paradox if transfer cells, which developed particularly in land plants, helped seagrasses to return to the sea.

The distinct ATPases that marine plants exploit in order to thrive in seawater have not yet been fully characterized because of the extreme difficulty in isolating cellular membranes from these plants. It would be of particular interest to determine whether the plasma-membrane ATPase induced in seawater-resistant seagrasses is distinct from that of terrestrial plants. It has been established that higher plants have no K^+/Na^+-ATPase that acts as an antiporter across the plasma membrane (for reviews, see Sze 1985; Nelson 1988). Using a cytochemical procedure, however, we found that the high ATPase activity in the cell membrane of seagrass leaf epidermis was quite sensitive to ouabain (Fig. 4B). This reagent is a specific inhibitor of K^+/Na^+-ATPases, without side effects, in animal cells (Jørgensen 1982) and, as far as we know, there have been no convincing reports of inhibition by this reagent of plant membrane ATPases. However, a probe specific for the mRNA of an animal K^+/Na^+-ATPase did not detect any such mRNA in seagrass leaf cells (not shown).

The H^+-translocating ATPase has been widely accepted as the major ion pump in membranes of higher-plant cells. This enzyme is responsible for creating an electrochemical proton gradient which is used for the transport of ions and nutrients that is mediated secondarily by specific carriers or channels (for reviews, see Sze 1985; Nelson 1988). The remarkably elevated levels of a putative mRNA for plasma-membrane H^+-ATPase during the development of seagrass leaves (Fig. 3) suggest that the H^+-ATPase is responsible for translocating ions across the invaginated cell membranes of seagrass leaf cells. In roots and old leaves of the halophyte *Atriplex nummularia,* expression of the gene(s) for plasma-membrane H^+-ATPase is significantly up-regulated with environmental salt (Niu et al. 1993a).

In plasma membrane isolated from most terrestrial plant cells, H^+-ATPase activity dependent on Mg^{2+} has been reported to be stimulated by K^+ and to be inhibited effectively by Ca^{2+} (for reviews, see Sze 1985; Nelson 1988). However, plasma-membrane ATPase activity dependent on both Ca^{2+} and Mg^{2+} has been demonstrated in some higher-plant cells (Winter-Sluiter et al. 1977; Chauhan and Lar 1980; Blumwald and Poole 1985; Evert et al. 1988; Brüggemann and Janiesch 1989; Sanchez-Aguayo et al. 1991), in particular by lead precipitation at an alkaline pH. No inhibitory effects of Ca^{2+} were observed also in our cytochemical experiments with the seagrass ATPase (Fig. 4C); Ca^{2+} could substitute completely for Mg^{2+} in the reaction medium. We also found a similar effect of Ca^{2+} on the ATPase activity in transfer cells from some terrestrial plants (not shown).

Thus, the plasma-membrane ATPase responsible for salinity tolerance in seawater-resistant seagrass cells cannot yet be designated as unusual, in spite of the noteworthy substitution of Ca^{2+} for Mg^{2+} and the specific inhibition of the enzymatic activity by ouabain in our experiments that involved lead precipitation. These cytochemical results should be re-examined after cloning of all the genes for the cell membrane-bound ATPases together with biochemical characterization using isolated vesicles from the invaginated plasma membranes.

The authors are grateful to Dr. P. Park of Teikyo University, School of Medicine, and Dr. K. Kasamo, National Institute for Food Sciences, Tsukuba, for their helpful advice. This work was supported by the Japanese Salt Science Research Foundation (No. 9228).

References

Arai, M., Pak, J.Y., Nomura, K., Nitta, T. (1991) Seawater-resistant, nonspherical protoplasts from seagrass leaves. Physiol. Plant. **83**, 551–559

Barnabas, A.D., Butler, V., Steinke, T.D. (1977) *Zostera capiensis* Setchell. I. Observations on the fine structure of the leaf epidermis. Z. Pflanzenphysiol. **85**, 417–427

Barnabas, A.D., Butler, V., Steinke, T.D. (1982) *Zostera capiensis* Setchell. III. Some aspects of wall ingrowth development in leaf blade epidermal cells. Protoplasma **110**, 87–94

Bentwood, B.J., Cronshaw, J. (1978) Cytochemical localization of adenosine triphosphatase in the phloem of *Pisum sativum* and its relation to the function of transfer cells. Planta **140**, 111–120

Birch, W.R. (1974) The unusual epidermis of the marine angiosperm *Halophila* Thou. Flora **163**, 410–414

Blumwald, E., Poole, R.J. (1985) Na^+/H^+ antiport in isolated tonoplast vesicles from storage tissue of *Beta vulgaris*. Plant Physiol. **75**, 163–167

Borowitzka, M.A., Vesk, M. (1978) Ultrastructure of the Corallinaceae. I. The vegetative cells of *Corallina offcinalis* and *C. cuvierii*. Mar. Biol. **46**, 295–304

Boutry, M., Michelet, B., Goffeau, A. (1989) Molecular cloning of a family of plant genes encoding a protein homologous to plasma membrane H^+-translocating ATPases. Biochim. Biophys. Res. Commun. **162**, 567–574

Braun, Y., Hassidim, M., Lerner, H.R., Reinhold, L. (1986) Studies on H^+-translocating ATPases in plants of varying resistance to salinity. I. Salinity during growth modulates the proton pump in the halophyte *Atriplex nummularia*. Plant Physiol. **81**, 1050–1056

Bressan, G., Ghirardelli, L.A., Bellemo, A. (1981) Research on the genus *Fosliella* (Rhodophyta, Corallinaceae): Structure, ultrastructure and function of transfer cells. Bot. Mar. **14**, 503–508

Brüggemann, W., Janiesch, P. (1989) Comparison of plasma membrane ATPase from salt-treated and salt-free grown *Plantago maritima* L. J. Plant Physiol. **134**, 20–25

Chauhan, E., Lar, M. (1980) Histochemical and ultrastructural investigations on the root of the moss *Physcomitrium cyathicarpum* Mitt. Evidence for the reservation of a Ca^{2+}-activated ATPase. J. Cytol. Genet. **15**, 191–195

Chauhan, E., Cowan, D.S., Hall, J.L. (1991) Cytochemical localization of plasma membrane ATPase activity in plant cells. Protoplasma **165**, 27–36

Chomeczynski, P., Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-RhOH-chloroform extraction. Anal. Biochem. **162**, 156–159

DeWitt, N.D., Harper, J.F., Sussman, M.R. (1991) Evidence for a plasma membrane proton pump in phloem cells of higher plants. Plant J. **1**, 121–128

Doohan, M.E., Newcomb, E.H. (1976) Leaf ultrastructure and $\delta^{13}C$ values of three seagrasses from the Great Barrier Reef. Aust. J. Plant Physiol. **3**, 9–23

Erdei, L., Stuiver C.E.E., Kuiper, P.J.C. (1980) The effect of salinity on lipid composition and on activity of Ca^{2+}- and Mg^{2+}-stimulated ATPases in salt-sensitive and salt-tolerant *Plantago* species. Physiol. Plant. **49**, 315–319

Evert, R.F., Mierzwa, R.J., Echerich, W. (1988) Cytochemical localization of phosphatase activity in vascular bundles and contiguous tissues of the leaf of *Zea mays* L. Protoplasma **146**, 41–51

Ewing, N.N., Wimmers, L.E., Meyer, D.J., Bennet, A.B. (1990) Molecular cloning of tomato plasma membrane H^+-ATPase. Plant Physiol. **94**, 1874–1881

Folsom, M.W., Cass, D.D. (1986) Changes in transfer cell distribution in the ovule of soybean after fertilization. Can. J. Bot. **64**, 965–971

Garbarino, J., Dupont, F.M. (1988) NaCl induces a Na^+/H^+ antiport in tonoplast vesicles from barley roots. Plant Physiol. **86**, 231–236

Graves, J.S., Gutknecht, J. (1977) Chloride transport and the membrane potential in the marine alga, *Halicystis parvula*. J. Membr. Biol. **36**, 65–81

Gunning, B.E.S. (1977) Transfer cells and their roles in transport of solutes in plants. Sci. Prog. Oxf. **64**, 539–568

Gunning, B.E.S., Pate, J.S. (1969) "Transfer cells". Plant cells with wall ingrowths, specialized in relation to short distance transport of solutes. Their occurrence, structure, and development. Protoplasma **68**, 107–133

Hall, J.L., Browning, A.J., Harvey, D.M.R. (1980) The validity of the lead precipitation technique for the localization of ATPase activity in plant cells. Protoplasma **104**, 193–200

Harper, J.F., Surowy, T.K., Sussman, M.R. (1989) Molecular cloning and sequence of cDNA encoding the plasma membrane proton pump (H^+-ATPase) of *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA. **86**, 1234–1238

Ikeda, M., Schmid, R., Oesterhelt, D. (1990) A Cl^- translocating adenosinetriphosphatase in *Acetabularia acetabulum*. I. Purification and characterization of a novel type of adenosinetriphosphatase that differs from chloroplast F_1 adenosinetriphosphatase. Biochemistry **29**, 2057–2065

Jagels, R. (1973) Studies of a marine grass, *Thalassia testudinum*. I. Ultrastructure of the osmoregulatory leaf cells. Am. J. Bot. **60**, 1003–1009

Jagels, R. (1983) Further evidence for osmoreguration in epidermal leaf cells of seagrasses. Am. J. Bot. **70**, 327–333

Jones, M.G.K., Northcote, D.H. (1972) Nematode-induced syncytium – A multinucleate transfer cell. J. Cell Sci. **10**, 789–809

Jørgensen, P.L. (1982) Mechanism of the Na^+, K^+ pump. Protein structure and conformations of the pure ($Na^+ + K^+$)-ATPase. Biochim. Biophys. Acta **694**, 27–68

Katz, D.B., Susmann, M.R., Mierzwa, R.J., Evert, R.F. (1988) Cytochemical localization of ATPase activity in oat roots localizes a plasma membrane-associated soluble phosphatase, not the proton pump. Plant Physiol. **86**, 841–847

Kramer, D. (1978) Transfer cells in the epidermis of roots of the Halophyte *Atriplex hastata* L. Naturwissenschaften **65**, 339

Kramer, D., Römheld, V., Landsberg, E. Ch., Marschner, H. (1980) Induction of transfer-cell formation by iron deficiency in the root epidermis of *Helianthus annuus* L. Planta **147**, 335–339

Kuo, J., Aioi, K., Iizumi, H. (1988) Comparative leaf structure and its functional significance of *Phyllospadix iwatensis* Makino and *Phyllospadix japonicus* Makino (Zosteraceae). Aquat. Bot. **30**, 169–187

Kuo, J., Ridge, R.W., Lewis, S.V. (1990) The leaf internal morphology and ultrastructure of *Zostera muelleri* Irmisch ex Aschers. (Zosteraceae): a comparative study of the intertidal and subtidal forms. Aquat. Bot. **36**, 217–236

Leopold, A.C., Willing, R.P. (1984) Evidence for toxicity effects of salt on membranes. In: Salinity tolerance in plants, pp. 67–76, Staples, R.C., Toenniessen, G.H., eds. John Wiley & Sons, New York

Lüttge, U. (1971) Structure and function of plant glands. Annu. Rev. Plant Physiol. **22**, 23–44

Maier, K., Maier, J. (1972) Localization of beta-glycerophosphatase and Mg^{2+}-activated adenosine triphosphatase in a moss haustorium, and the relation of these enzymes to the cell wall labyrinth. Protoplasma **75**, 91–112

Nelson, N. (1988) Structure, function, and evolution of proton-ATPases. Plant Physiol. **86**, 1–3

Niu, X., Narasimhan, M.L., Salzman, R.A., Bressan, R.A., Hasegawa, P.M. (1993a) NaCl regulation of plasma membrane H^+-ATPase gene expression in a glycophyte and halophyte. Plant Physiol. **103**, 713–718

Niu, X., Zhu, J.-K., Narasimhan, M.L., Bressan, R.A., Hasegawa, P.M. (1993b) Plasma-membrane H^+-ATPase gene expression is regulated by NaCl in cells of the halophyte *Atriplex nummularia* L. Planta **190**, 433–438

Okazaki, M. (1977) Some enzymatic properties of Ca^{2+}-dependent adenosine triphosphatase from a calcareous red alga, *Serraticardia maxima* and its distribution in marine algae. Bot. Mar. **20**, 347–354

Parets-Soler, A., Pardo, J.M., Serrano, R. (1990) Immunocytolocalization of plasma membrane H^+-ATPase. Plant Physiol. **93**, 1654–1658

Pate, J.S., Gunning, B.E.S., Milliken, F.F. (1970) Function of transfer cells in the nodal regions of stems, particularly in relation to the nutrition of young seedlings. Protoplasma **71**, 313–334

Rogers, S.O., Bendich, A.J. (1988) In: Plant molecular biology manual, pp. A6/1–A6/10, Gelvin, S.B., Schilperoort, R.A., eds. Kuluwer Academic Publishers, Dordrecht

Sanchez-Aguayo, I., Gonzarez-Utor, A. L., Medina, A. (1991) Cytochemical localization of ATPase activity in salt-treated and salt-free grown *Lycopersicon esculentum* roots. Plant Physiol. **96**, 153–158

Spurr, A.R. (1969) A low-viscosity embedding medium for electron microscopy. J. Ultrastruct. Res. **26**, 31–34

Sze, H. (1985) H^+-Translocating ATPases: advances using membrane vesicles. Annu. Rev. Plant Physiol. **36**, 175–208

Villalba, J.M., Lützelschwab, M., Serrano, R. (1991) Immunocytolocalization of plasma-membrane H^+-ATPase in maize coleoptiles and enclosed leaves. Planta **185**, 458–461

Wada, M., Satoh, S., Kasamo, K., Fujii, T. (1989) Presence of a Na^+-activated ATPase in the plasma membrane of the marine Raphidophycean *Heterosigma akashiwo*. Plant Cell Physiol. **30**, 923–928

Wada, M., Takano, M., Kasamo, K. (1992) Nucleotide sequence of a complementary DNA encoding plasma membrane H^+-ATPase from rice (*Oryza sativa* L.). Plant Physiol. **99**, 794–795

Walker, R.R., Leigh, R.A. (1981) Characterization of a salt-stimulated ATPase activity associated with vacuoles isolated from storage roots of red beet (*Beta vulgaris* L.). Planta **153**, 140–149

Wimmers, L.E., Turgeon, R. (1991) Transfer cells and solute uptake in minor veins of *Pisum sativum* leaves. Planta **186**, 2–12

Winter-Sluiter, E., Läuchli, E.A., Kramer, D. (1977) Cytochemical localization of K^+-stimulated adenosine triphosphatase activity in xylem parenchymal cells of barley roots. Plant Physiol. **60**, 923–927

Yeung, E.C., Peterson, R.L. (1975) Fine structure during ontogeny of xylem transfer cells in the rhizome of *Hieracium floribundum*. Can. J. Bot. **53**, 432–438