Communication

Immunocytolocalization of Plasma Membrane H⁺-ATPase

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ABSTRACT

The localization of plasma membrane H⁺-ATPase has been studied at the optical microscope level utilizing frozen and paraffin sections of *Avena sativa* and *Pisum sativum*, specific anti-ATPase polyclonal antibody, and second antibody coupled to alkaline phosphatase. In leaves and stems the ATPase is concentrated at the phloem, supporting the notion that it generates the driving force for phloem loading. In roots the ATPase is concentrated at both the periphery (rootcap and epidermis) and at the central cylinder, including endodermis and vascular cells. This supports a 'two-pump' mechanism for ion absorption, involving active uptake at the epidermis, symplast transport across the cortex, and active efflux at the xylem. The low ATPase content of root meristem and elongation zone may explain the observed transorgan H⁺ currents, which leave nongrowing parts and enter growing tips.

The major ATPase of plant plasma membranes is a H⁺ pump, which seems to play a central role in plant physiology. The proton gradient generated by the enzyme is the driving force for active nutrient transport, and the pH changes resulting from proton pumping may be involved in growth control (21). At the level of whole plants, the loading of root xylem with inorganic nutrients and the loading of leaf phloem with organic nutrients seem to depend on active transport processes driven by the H⁺-ATPase (3, 12, 16).

Knowledge about the distribution of plasma membrane H⁺-ATPase in plant tissues is essential for understanding the pathway and mechanism of nutrient transport (3, 12, 16). Previous approaches to this problem consisted of the cytochemical staining for ATP hydrolysis by lead-induced precipitation of the released Pi (7, 25–27). However, it has recently been demonstrated that the plasma membrane H⁺-ATPase is inactivated by both the lead and the fixatives used in the cytochemical procedure and that the measured ATP hydrolysis is catalyzed by a molybdate-sensitive phosphatase (13). Clearly, a more specific approach to ATPase localization was needed.

We have expressed in *Escherichia coli* the carboxyl-terminal domain of a cloned ATPase gene (18) and generated specific polyclonal antibody against the enzyme. By utilizing this antibody and either frozen or paraffin sections of plant tissues, we found that the ATPase is highly enriched in vascular tissues

and root epidermis and endodermis. The implications of this distribution for current models of nutrient transport in plants are discussed.

MATERIALS AND METHODS

Plant Material

Oats (*Avena sativa*) and peas (*Pisum sativum*) were local varieties distributed in Heidelberg by the Zentralgenossenschaft für Landwirtschaftliche Erzeugnisse. They were grown for 2 weeks in vermiculite. The plant chamber was maintained at 23°C with a regime of 16 h day-8 h night.

Generation of Antibody

The carboxyl-terminal domain of the ATPase gene was obtained from the cDNA clone (18) as a *Bst*NI fragment of 427 base pairs (nucleotides 2915–3342). It was blunt-ended with the Klenow fragment of DNA polymerase and subcloned with the right orientation into the *Sma*I site of the expression vector pEX3 (22). This produced an in-frame fusion of the *cro-lacZ* gene with the coding region for amino acids 851–949. Purification of the fusion protein from the inclusion bodies of the bacteria and rabbit immunization were performed as described in the PEXFIT manual (Genofit, Geneva, Switzerland).

Membrane and Enzyme Preparation

Oat roots were homogenized as described (20), and a crude membrane fraction was prepared by centrifugation of the homogenate during 1 h at 40,000 rpm (Beckman rotor 70 Ti). The plasma membrane ATPase was purified to near homogeneity as described (20).

Electrophoresis and Blotting

PAGE in SDS, transfer to nitrocellulose, and immunodetection were as described (1). Samples were first precipitated with TCA before dissolving in the SDS buffer and heating was limited to 37°C. These modifications were needed to prevent both proteolytic degradation and aggregation of the ATPase (20). Nonfat dried milk was used in the blocking of the nitrocellulose in addition to Tween 20 (1). Preimmune and immune sera were diluted 1/2000 and the second antibody conjugated to alkaline phosphatase (Promega, Madison, anti-rabbit IgG alkaline phosphatase conjugate developed in goat and affinity purified, 1 mg/mL) was diluted 1/5000.

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Immunocytolocalization

Frozen sections of 14 to 20 μm were prepared at −22°C in a cryostat and picked up on subbed slides as described (11, 17). Sections were air-dried and dehydrated at −20°C, first with 40% ethanol and then with 55% ethanol. They were finally fixed at −20°C with 75% ethanol-25% acetic acid, washed at room temperature with 75% ethanol, and air-dried. Paraffin sections of 10 μm were made as described (15), except that the tissue was fixed with 2% paraformaldehyde before dehydration.

All the incubations and washes were done by overlaying tissue sections with the corresponding solutions. The basic medium was TBS[2] buffer containing 2% nonfat dried milk and either 0.05% (blocking and antibody dilutions) or 0.5% (washes) Tween 20. Sections were blocked by 30 min incubation in basic medium. Preimmune and immune sera were diluted 1/500 (frozen sections) or 1/250 (paraffin sections) and incubated 3 h with the sections. After 3 × 10 min washes, second antibody conjugated to alkaline phosphatase (see above) was diluted 1/100 and incubated 2 h with the sections. After 3 × 10 min washes, the reaction of alkaline phosphatase was developed for 30 min as described (1).

A Nikkon Diaphot microscope was utilized for sample visualization and photography. To optimize differential visualization of the alkaline phosphatase stain with respect to the contrast of the tissue, the condenser had to be moved away from the sample to provide a low intensity of diffused light. This produced some blurring of the image. All samples (preimmune controls and immunodecorated sections) were photographed under identical conditions.

RESULTS

Antibody has been generated against the last 99 amino acids of one of the three ATPase genes of *Arabidopsis thaliana* (18). It reacts with purified plasma membrane ATPase from oat roots (Fig. 1, lanes 3 and 7) and with plasma membrane ATPases from tobacco, sunflower, and corn (R Serrano, unpublished data). This carboxyl-terminal domain has only 19 to 20 amino acid changes with respect to other isoforms of *Arabidopsis* ATPase (10, 18) and 36 changes with respect to one of the isoforms of tobacco ATPase (2). Therefore, most of the amino acids in this region are highly conserved. The specificity of the antiserum is demonstrated by the fact that in crude membranes showing many protein bands only the ATPase band of 100 kD is decorated (Fig. 1, lanes 2 and 6).

We have utilized frozen (11, 17) and paraffin (15) sections for immunolocalization studies because they preserve better the antigenicity of tissue proteins than resin-embedded sections (8, 11). Structural preservation, however, is not always good with these methods (8, 11), and we have encountered most difficulties with root tissues. Figure 2A shows a longitudinal frozen section of a root tip. Cellular detail is poor and no improvement could be obtained by using paraffin sections. However, despite its low resolution, this picture clearly indicates enrichment of the ATPase at the root periphery (rootcap and epidermis) and at the central cylinder. The meristematic
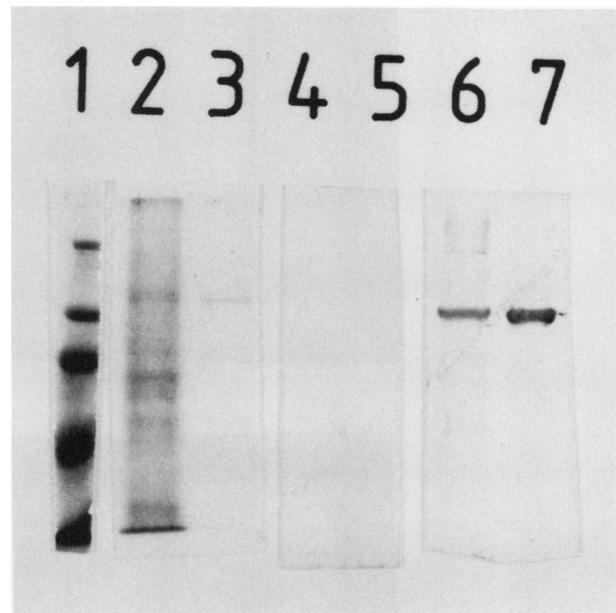
[2] Abbreviation: TBS, 0.15 M NaCl and 20 mM Tris-HCl (pH 8.0).

Figure 1. Specificity of antibody in Western blots. Samples of 20 μg crude membranes (lanes 2, 4, and 6) or 2 μg purified ATPase (lanes 3, 5, and 7) were separated by electrophoresis and either stained for protein with Coomassie blue R-250 (lanes 2 and 3) or transferred to nitrocellulose and decorated with either preimmune serum (lanes 4 and 5) or antiserum (lanes 6 and 7). Lane 1: Molecular mass standards of 200, 98, 68, 43, and 26 kD.

and elongation zones and the cortex of differentiated zones contain relatively little ATPase. Transverse sections at the meristematic zone (Fig. 2B) also show poor cellular detail but confirm the peripheral enrichment of the ATPase (at the rootcap under the mucilage layer). Much better structural detail was obtained in transverse sections of the differentiation zone (Fig. 2C), where the external part of the epidermal layer and the central cylinder are intensively labeled. A mature central cylinder surrounded by cortical cells is shown in Figure 2D. Intense labeling of endodermis, pericycle, and vascular cells is observed. The root surface was also labeled in this differentiated part of the root but structural preservation was very poor and we could not ascertain if the stain corresponded to the epidermis alone or to epidermis and exodermis (not shown).

Figure 3A (low magnification) shows dark spots corresponding to staining of leaf veins. Figure 3B (high magnification) demonstrates specific staining at the phloem layer of a vascular bundle. In stems (Fig. 3C) the phloem of collateral and bicollateral vascular bundles (the later with internal and external phloem [6]) is specifically stained.

DISCUSSION

The enrichment of ATPase at the phloem of leaf veins supports a role for the enzyme in phloem loading, as suggested from biochemical and physiological studies (12, 16). At the optical microscope level we cannot definitively ascertain the different types of cells labeled by antibody. In addition to
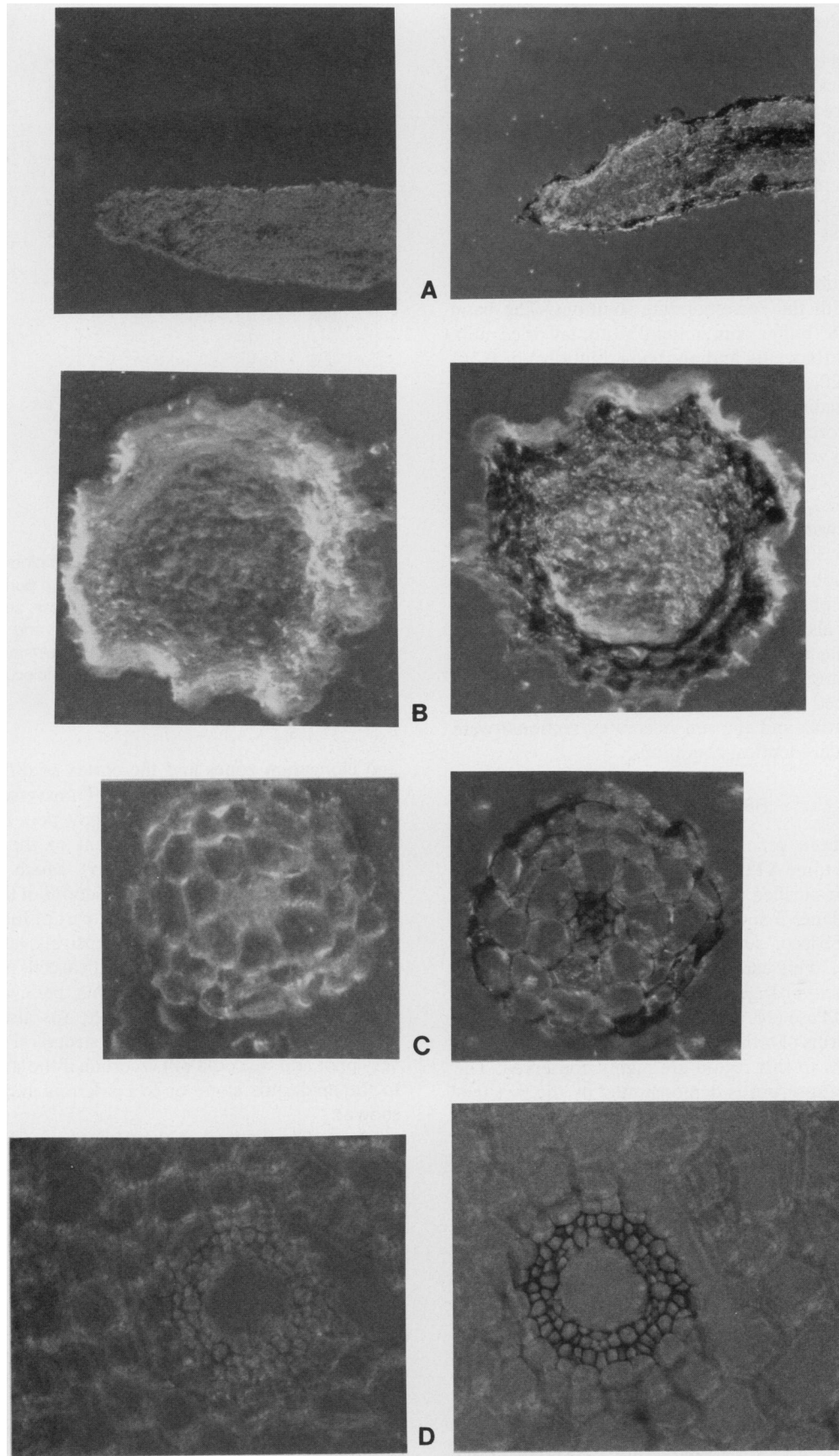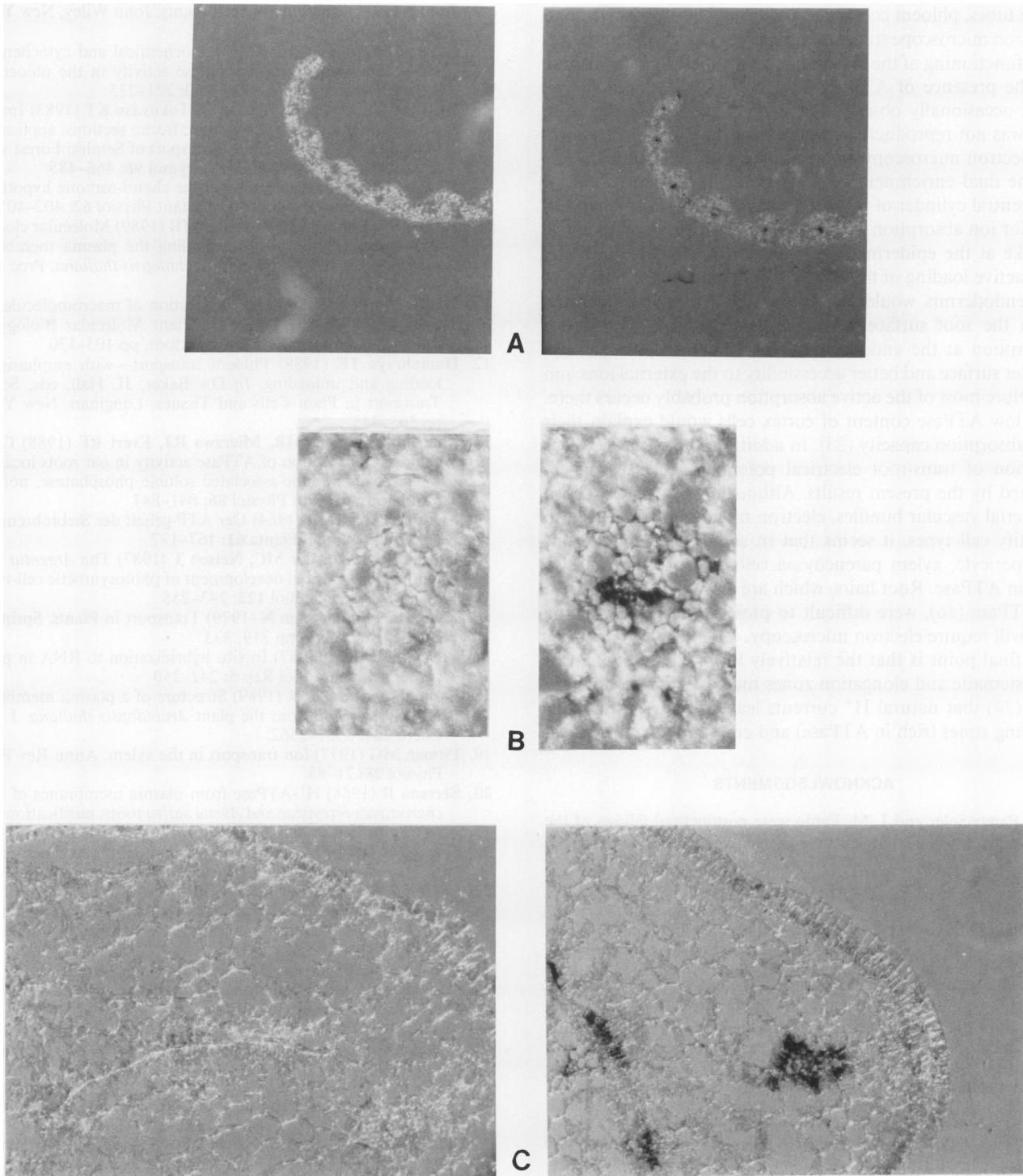
Figure 3. Immunocytolocalization of ATPase in paraffin sections of oat leaves (A and B) and in frozen sections of pea stems (C). A, Overview of a leaf at low magnification (×20); B, detail of a vein (×370); C, portion of a stem section (×100). Sections were decorated with either preimmune serum (left) or antiserum (right).

Figure 2. Immunocytolocalization of ATPase in frozen sections of oat roots. Longitudinal sections from root tips (A, ×100) and transverse sections of meristematic zone (B, ×200), differentiation zone (C, ×260), and mature central cylinder (D, ×300) were decorated with either preimmune serum (left) or antiserum (right).

sieve tubes, phloem companion cells may also be labeled and electron microscope studies are under way to clarify this point. The functioning of the ATPase in sieve tubes is made possible by the presence of ATP in phloem sap (14). Although we have occasionally observed staining of stomata guard cells, this was not reproducible and we want to confirm this point by electron microscopy before reaching any conclusion.

The dual enrichment of ATPase at the epidermis and at the central cylinder of roots supports the 'two-pump' hypothesis for ion absorption (3, 4, 9, 16, 19), which assumes active uptake at the epidermis, symplasmic transport to the stele, and active loading of the xylem. The high ATPase content of the endodermis would make plausible an apoplastic route from the root surface to the central cylinder, with active absorption at the endodermis. However, the epidermis has greater surface and better accessibility to the external ions and therefore most of the active absorption probably occurs there. The low ATPase content of cortex cells would explain their low absorption capacity (23). In addition, the bi-phasic composition of trans-root electrical potentials (5) is easily explained by the present results. Although, as indicated above for aerial vascular bundles, electron microscopy is needed to identify cell types, it seems that in addition to endodermis and pericyle, xylem parenchyma cells and phloem are also rich in ATPase. Root hairs, which are also probably enriched in ATPase (16), were difficult to preserve during sectioning and will require electron microscopy.

A final point is that the relatively low ATPase content of meristematic and elongation zones may explain the observation (24) that natural H^+ currents leave differentiated, nongrowing zones (rich in ATPase) and enter growing tips.

ACKNOWLEDGMENTS

LITERATURE CITED

1. **Blake MS, Johnston KH, Russell-Jones GJ, Gotschlich EC** (1984) A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. Anal Biochem **135:** 175–179

2. **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. Biochem Biophys Res Commun **162:** 567–574

3. **Clarkson DT** (1988) Movements of ions across roots. *In* DA Baker, JL Hall, eds, Solute Transport in Plant Cells and Tissues. Longman, New York, pp 251–304

4. **Clarkson DT, Hanson JB** (1986) Proton fluxes and the activity of a stelar proton pump in onion roots. J Exp Bot **37:** 1136–1150

5. **De Boer AH, Prins HBA, Zanstra PE** (1983) Bi-phasic composition of trans-root electrical potential in roots of *Plantago* species: involvement of spatially separated electrogenic pumps. Planta **157:** 259–266

6. **Esau K** (1977) Anatomy of Seed Plants. John Wiley, New York, pp 308–309

7. **Gilder J, Cronshaw J** (1974) A biochemical and cytochemical study of adenosine triphosphatase activity in the phloem of *Nicotiana tabacum.* J Cell Biol **60:** 221–235

8. **Griffiths G, Simons K, Warren G, Tokuyasu KT** (1983) Immunoelectron microscopy using thin, frozen sections: application to studies of the intracellular transport of Semliki Forest virus spike glycoproteins. Methods Enzymol **96:** 466–485

9. **Hanson JB** (1978) Application of the chemi-osmotic hypothesis to ion transport across the root. Plant Physiol **62:** 402–405

10. **Harper JF, Surowy TK, Sussman MR** (1989) Molecular cloning and sequence of a cDNA encoding the plasma membrane proton pump (H^+-ATPase) of *Arabidopsis thaliana.* Proc Natl Acad Sci USA **86:** 1234–1238

11. **Hawes C** (1988) Subcellular localization of macromolecules by microscopy. *In* CH Shaw, ed, Plant Molecular Biology. A Practical Approach. IRL Press, Oxford, pp 103–130

12. **Humphreys TE** (1988) Phloem transport—with emphasis on loading and unloading. *In* DA Baker, JL Hall, eds, Solute Transport in Plant Cells and Tissues. Longman, New York, pp 305–345

13. **Katz DB, Sussman MR, Mierzwa RJ, Evert RF** (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

14. **Kluge M, Ziegler H** (1964) Der ATP-gehalt der Siebrohrensafte von Laubbaumen. Planta **61:** 167–177

15. **Langdale JA, Metzler MC, Nelson T** (1987) The *Argentia* mutation delays normal development of photosynthetic cell-types in *Zea mays.* Dev Biol **122:** 243–255

16. **Lüttge U, Higinbotham N** (1979) Transport in Plants. Springer-Verlag, New York, pp 319, 333

17. **Meyerowitz, EM** (1987) In situ hybridization to RNA in plant tissue. Plant Mol Biol Rep **5:** 242–250

18. **Pardo JM, Serrano, R** (1989) Structure of a plasma membrane H^+-ATPase gene from the plant *Arabidopsis thaliana.* J Biol Chem **264:** 8557–8562

19. **Pitman MG** (1977) Ion transport in the xylem. Annu Rev Plant Physiol **28:** 71–88

20. **Serrano R** (1988) H^+-ATPase from plasma membranes of *Saccharomyces cerevisiae* and *Avena sativa* roots: purification and reconstitution. Methods Enzymol **157:** 533–544

21. **Serrano R** (1989) Structure and function of plasma membrane ATPase. Annu Rev Plant Physiol Plant Mol Biol **40:** 61–94

22. **Stanley KK, Luzio JP** (1984) Construction of a new family of high efficiency bacterial expression vectors: identification of cDNA clones coding for human liver proteins. EMBO J **3:** 1429–1434

23. **Van Iren F, Boers-van der Sluijs P** (1980) Symplasmic and apoplasmic radial ion transport in plant roots. Planta **148:** 130–137

24. **Weisenseel MH, Dorn A, Jaffe LF** (1979) Natural H^+ currents traverse growing roots and root hairs of barley (*Hordeum vulgare* L.). Plant Physiol **64:** 512–518

25. **Williams L, Hall JL** (1987) ATPase and proton pumping activities in cotyledons and other phloem-containing tissues of *Ricinus communis.* J Exp Bot **38:** 185–202

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

27. **Yapa PAJ, Spanner DC** (1974) Localization of adenosine triphosphatase activity in mature sieve elements of *Tetragonia.* Planta **117:** 321–328