BIOLOGIA PLANTARUM 39 (2): 169-177, 1997

Stimulation of H^+-transport activity of vacuolar H^+-ATPase by activation of H^+-PPase in *Kalanchoë blossfeldiana*

E. FISCHER-SCHLIEBS, J.-B. MARIAUX*, and U. LÜTTGE

Institut für Botanik, Technische Hochschule Darmstadt, Schnittspahnstrasse 3-5, D-64287 Darmstadt, Germany
*Max Planck Institut für Züchtungsforschung, Carl-von Linné-Weg 10, D-50829 Köln, Germany**

Abstract

In *Kalanchoë blossfeldiana* cv. Tom Thumb the initial rate of ATP-dependent H^+-transport into tonoplast vesicles was stimulated up to three times if the H^+-ATPase (EC 3.6.1.3) was energized a few minutes after pre-energization of the H^+-PPase (EC 3.6.1.1). H^+-PPase-activated ATP-dependent H^+-transport was observed in plants of *K. blossfeldiana* cultivated in short day (SD) or long day (LD) conditions expressing different degrees of crassulacean acid metabolism (CAM). However, based on the higher activity and protein amount of H^+-PPase and H^+-ATPase present in the vacuolar membrane of SD plants the maximum H^+-transport activity in the stimulated mode of the H^+-ATPase was significantly higher in tonoplast vesicles of SD plants than of LD plants. Hence, a co-ordinated action of the H^+-PPase and H^+-ATPase at the tonoplast of *Kalanchoë* could allow a higher transport capacity at the vacuolar membrane when plants perform high CAM. Immunoprecipitation experiments with an antiserum raised against the A-subunit of the vacuolar H^+-ATPase of *Mesembryanthemum crystallinum* L. showed that in SD and LD plants of *K. blossfeldiana* the H^+-PPase was co-precipitated with the vacuolar H^+-ATPase holoenzyme. The co-percipitation of the two transport proteins indicates a close structural localization of the H^+-PPase and the A-subunit of the vacuolar H^+-ATPase.

Additional key words: Crassulacean acid metabolism, photoperiodism, proton transport, tonoplast.

Received 21 June 1996, *accepted* 5 September 1996.

Abbreviations: CAM - Crassulacean acid metabolism; LD - long day; PP_i - inorganic pyrophosphate; SD - short day; SDS-PAGE - sodium dodecyl sulphate gel electrophoresis.

Acknowledgements: We thank R. Ratajczak for stimulating discussion. This work was supported by the Deutsche Forschungsgemeinschaft (Bonn, Germany) in the frame of the Sonderforschungsbereich 199 (project B2).

Fax: (+49 6151) 16 4630, e-mail: Fischer-Schliebs@Bio1.Bio.TH-Darmstadt.De.

Introduction

The vacuolar H^+-PPase (EC 3.6.1.1) is an important component of the tonoplast of plant cells (for review see Leigh *et al.* 1994). It has been reported to generate an inside positive electrochemical proton gradient of similar magnitude as is established by the vacuolar H^+-ATPase (EC 3.6.1.3) on the same membrane (Johannes and Felle 1990). Thus, beside the H^+-ATPase the H^+-PPase may contribute to the energetization of a broad range of H^+-coupled and electrically coupled transport processes at the vacuolar membrane of higher plant cells. However, up to now it is not clearly understood, why two independent H^+-translocating enzymes, hydrolyzing ATP or PP_i, are needed at the tonoplast. According to one idea the H^+-PPase may indeed conserve the free energy of PP_i generated in synthetic reactions in the form of an electrochemical proton gradient at the tonoplast, and hence, may play an important role in regulating the cytoplasmic PP_i level (Wang *et al.* 1986, Hoffmann and Bentrup 1989, Rea and Poole 1993).

The idea that the H^+-PPase alternatively to the H^+-ATPase might energize the tonoplast could be of importance in plants performing Crassulacean acid metabolism (CAM) where a high capacity for the nocturnal secondary active transport of organic acids, mainly malic acid, into the vacuole is required and the availability of ATP is restricted (Lüttge *et al.* 1981, Lüttge 1987). However, for thermodynamic reasons due to the steep H^+-gradients established at the tonoplast during the night and the lower free energy of PP_i hydrolysis with respect to ATP it seems unlikely that the H^+-PPase contributes much to acid accumulation in CAM plants. Nevertheless, it was shown for the CAM plant *Kalanchoë daigremontiana* that the activation of the H^+-PPase by its substrate magnesium pyrophosphate kinetically enhanced H^+-transport across the membrane of isolated tonoplast vesicles by the H^+-ATPase if the latter was activated by its substrate MgATP a few minutes later (Marquardt-Jarczyk and Lüttge 1990). The stimulation of the H^+-ATPase by the H^+-PPase indicated a co-ordinated action of the two H^+-translocating tonoplast proteins which may be relevant in *Kalanchoë* species for the functioning of CAM.

In leaves of the CAM plant *K. blossfeldiana* cv. Tom Thumb the degree of CAM expression is promoted by exposing plants to short day conditions (SD) with a photoperiod of less than 10 h (Brulfert *et al.* 1982). Thus, *K. blossfeldiana* can serve as a model system to investigate the impact of CAM on the interaction of the H^+-PPase and H^+-ATPase in the vacuolar membrane. The aim of our study was to gain more information on a possible co-operative relationship of the two membrane proteins in another *Kalanchoë* species and the influence of the degree of CAM expression on this relationship.

Materials and methods

Plants of *Kalanchoë blossfeldiana* cv. Tom Thumb were grown from seeds in the glasshouse in soil (day/night temperature: 25 - 30/13 - 20 °C, 16-h photoperiod). When plants were 2 month old, they were transferred to a phytotron and exposed for

42 d to either SD conditions (8 h light/16 h darkness) to enhance CAM or LD conditions (16 h light/8 h darkness) at an irradiance of 350 - 400 µmol(PAR) m^{-2} s^{-1} and day/night temperature of 24/15 °C, and relative humidity of 60/80 %.

Malate analysis: Leaf samples of SD and LD plants were collected from the 4th and 5th leaf pair at the ends of light and dark phases, respectively. Leaves were numbered from the apex. The malate concentration of the mesophyll cell sap was determined according to Hohorst (1970).

Preparation of tonoplast vesicles: Tonoplast vesicles were prepared from *K. blossfeldiana* leaves according to Fischer-Schliebs *et al.* (1997). For membrane isolation 30 g of fresh mass of the 4th to 6th leaf pair were used.

Measurements of PP_i and ATP hydrolysis: Pyrophosphate- and ATP-hydrolysis was determined following the rate of P_i released as described by Fischer-Schliebs *et al.* (1997). The final reaction volume was 0.225 cm^3 including 1 to 2 µg membrane protein. The PP_i-hydrolytic activity of the vacuolar H^+-PPase refers to the K^+-stimulated PP_i hydrolysis. The ATP-hydrolytic activity of the H^+-ATPase refers to the NO_3^--sensitive ATP hydrolysis.

Measurements of H^+-transport activity: The formation of a pH gradient across the membrane vesicles was measured following the fluorescence quenching of the dye quinacrine (Marquardt-Jarczyk and Lüttge 1990). Measurements were performed at 25 °C with a *Sigma ZWS II* photometer equipped with a fluorescence attachment using an excitation wavelength of 427 nm. Emission was measured through an interference filter at 530 nm. The standard reaction mixture (final volume: 1 cm^3) contained: 25 mM bis-tris propane/MES, pH 8.0, 250 mM saccharose, 1.5 mM dithiothreitol, 3 µM quinacrine, and 20 to 50 µg membrane protein. The initial rate of H^+-transport activity is given as the relative fluorescence quenching [% Q $µg^{-1}$(protein) min^{-1}].

Sodium dodecyl sulphate gel electrophoresis: SDS-PAGE was performed as described by Ratajczak *et al.* (1994a) on a gradient gel containing 10 to 15 % total acrylamide using a Laemmli buffer system (Laemmli 1970). Silver staining was performed according to Oakley *et al.* (1980).

Immunoprecipitation: Solubilization of tonoplast protein with octyl glucoside and immunoprecipitation was carried out as described by Ratajczak *et al.* (1994b) with some modifications (Fischer-Schliebs *et al.* 1996). The immunoprecipitation experiments were performed with a polyclonal antiserum directed against the A-subunit of the tonoplast H^+-ATPase of *M. crystallinum* (Ratajczak *et al.* 1994b). The amount of total tonoplast protein used for immunoprecipitation was 200 µg.

Protein determination: The protein content was determined according to Peterson (1977). Bovine serum albumin was used as a standard.

Results

The curves of quinacrine fluorescence quenching due to PP_i- or ATP-dependent H^+-transport activities of tonoplast vesicles of SD plants of *K. blossfeldiana* (Fig. 1) showed that the sum of transport rates obtained with each substrate alone was in the range of the rate obtained with both substrates added together at the same time. Thus, the initial rates of PP_i- and ATP-dependent H^+-transport into tonoplast vesicles were additive. Conversely, the PP_i- and ATP-dependent H^+-transport activities were non-additive in establishing the steady state level indicating that the final steady state was under control of a maximal attainable proton gradient determined through a balance between the activity of the H^+-transporting enzymes and the H^+-permeability of the membrane. The initial rates of the ATP-dependent H^+-transport were stimulated significantly to about 300 % when ATP was added a few minutes after pre-energization of vesicles with PP_i. Therefore, as in *K. daigremontiana* (Marquardt-Jarczyk and Lüttge 1990), in *K. blossfeldiana* an H^+-PPase-activated ATP-dependent H^+-transport could be distinguished from the basal PP_i-dependent and the basal ATP-dependent H^+-transport.

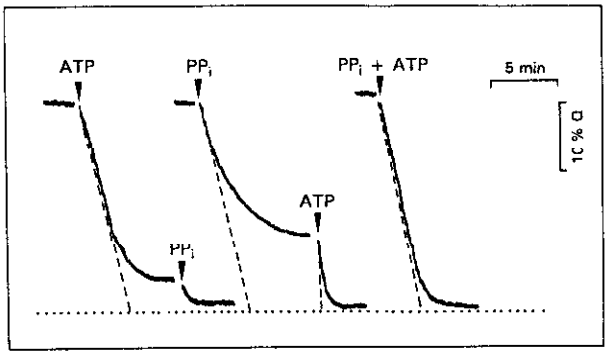

Fig. 1. Characteristic PP_i- and ATP-dependent quinacrine fluorescence quenching curves in the presence of 50 mM KCl at pH 8 and a temperature of 25 °C of one membrane preparation of *Kalanchoë blossfeldiana* cultivated under SD conditions. Additions to the reaction medium were as followed: ATP, 3 mM MgATP; PP_i, 0.2 mM Na_4PP_i + 2 mM $MgSO_4$; PP_i + ATP, 0.2 mM Na_4PP_i + 2 mM $MgSO_4$ + 3 mM MgATP. The *dashed tangents* indicate the slopes of curves and the *dotted lines* the maximal fluorescence quench attainable in one given membrane vesicle preparation.

The phenomenon of a H^+-PPase-stimulated ATP-dependent H^+-transport was observed in plants cultivated either in SD conditions which enhances CAM expression or LD conditions in which only a weak CAM is performed as indicated by the day-night change of malate concentration in the leaf cell sap (Table 1, Fig. 2). The relative stimulation of the ATP-dependent H^+-transport by an activation of the H^+-PPase was independent of the degree of CAM expression with a stimulation by a factor of 3.27 for SD plants and 2.94 for LD plants, respectively. In SD plants the rate of ATP-dependent H^+-transport obtained by addition of ATP after PP_i was

statistically significantly larger at the $P = 0.05$ % (Student's t-test) than that obtained with simultaneous addition of PP_i and ATP, while in LD plants the difference was not statistically significant. In SD and LD plants the hydrolytic activity of H^+-PPase and H^+-ATPase were in the same order of magnitude (Table 1). However, shifting

Table 1. Rates of PP_i and ATP hydrolysis, basal initial rates of PP_i-dependent and ATP-dependent H^+-transport activities of tonoplast vesicles, and day-night changes in malate levels (Δmalate) in the cell sap of SD and LD plants of *Kalanchoë blossfeldiana*. Data represent results of four repetitions of membrane preparations ± standard deviation. Data obtained from SD and LD plants are statistically different at the level of $P < 0.005$ (*a*) or $P < 0.05$ (*b*).

	Δ Malate [mM]	H^+-PPase [μmol mg^{-1} (protein) min^{-1}]	[% Q μg^{-1} (protein) min^{-1}]	H^+-ATPase [μmol mg^{-1} (protein) min^{-1}]	[% Q μg^{-1} (protein) min^{-1}]
LD plants	32.9 ± 6.7^a	0.039 ± 0.008^a (100 %)	0.019 ± 0.004^a (100 %)	0.041 ± 0.021^a (100 %)	0.026 ± 0.005^b (100 %)
SD plants	78.1 ± 8.2^a	0.178 ± 0.048^a (456 %)	0.096 ± 0.006^a (505 %)	0.182 ± 0.056^a (444 %)	0.063 ± 0.022^b (246 %)

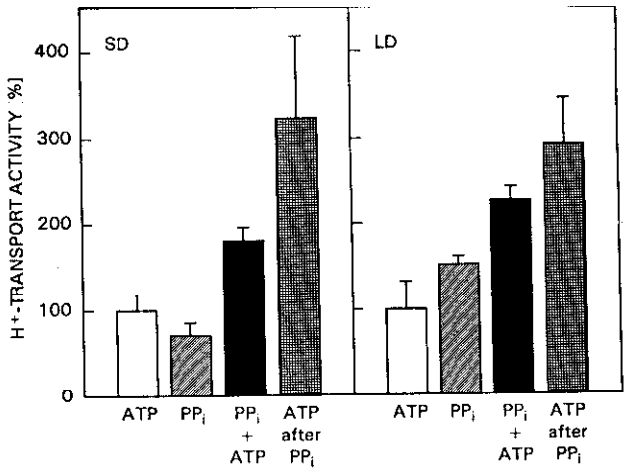

Fig. 2. Initial rates of H^+-transport activity with different regimes of substrate addition to tonoplast vesicles of *Kalanchoë blossfeldiana* from SD and the LD plants at day 42 after shortening the photoperiod; means of four membrane preparations ± standard deviation; for SD and LD plants 100 % was 0.063 ± 0.022 and 0.026 ± 0.005 % Q μg^{-1}(protein) min^{-1}, respectively.

plants from weak to high CAM was accompanied by a higher activity of both enzymes based on the presence of a higher amount of protein in the vacuolar membrane (Fischer-Schliebs *et al.* 1997; see also staining intensities of the H^+-PP_iase and subunits of the H^+-ATPase on the gel shown in Fig. 3) Therefore, although the relative stimulation of the ATP-dependent H^+-transport by activation of the H^+-PPase

was independent on the degree of CAM expressed the maximum absolute rate of the H^+-PPase-activated ATP-dependent H^+-transport activity related to total tonoplast protein of tonoplast vesicles from SD plants was with 0.185 ± 0.50 % Q μg^{-1}(protein) min^{-1} about two times higher than the maximum rate of the stimulated H^+-transport activity of the H^+-ATPase [0.085 ± 025 % Q μg^{-1}(protein) min^{-1}] of tonoplast vesicles of LD plants.

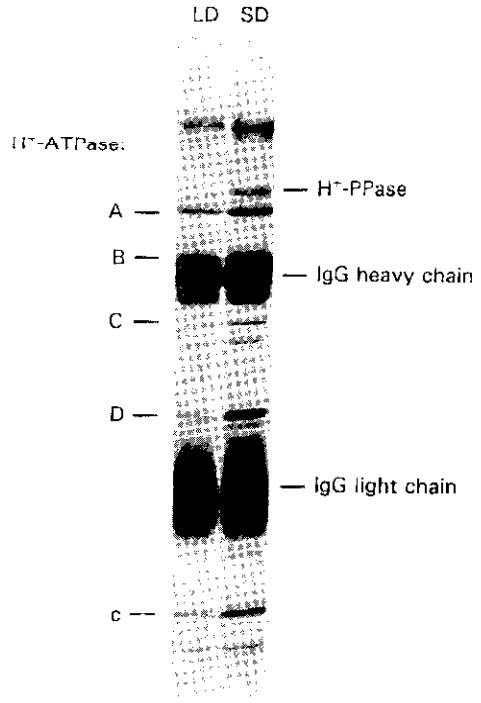

Fig. 3. Immunoprecipitation of the vacuolar H^+-PPase and H^+-ATPase of *Kalanchoë blossfeldiana* of tonoplast fractions of LD and SD plants. Silver stained polyacryamide gel (10 to 15 % total acrylamide) of tonoplast polypeptides immunoprecipitated by polyclonal antibodies directed against the subunit A of the tonoplast H^+-ATPase of *Mesembryanthemum crystallinum* coupled to protein A-*Sepharose*. Letters refer to subunits of the H^+-ATPase. The experiment was carried out with 200 µg octylglucoside-solubilized total tonoplast protein.

Immunoprecipitation of the holoenzyme of the vacuolar H^+-ATPase from octylglucoside-solubilized total tonoplast proteins of *K. blossfeldiana* was carried out with polyclonal antibodies raised against the subunit A of the H^+-ATPase of *M. crystallinum*. Subsequently performed SDS-PAGE showed that besides the subunits of the H^+-ATPase (as marked in Fig. 3) the 72 kDa polypeptide of the vacuolar H^+-PPase was co-precipitated revealing a close association of the two H^+-translocating membrane proteins. The immunoprecipitation of the H^+-PPase could not have occurred by a direct reaction of the H^+-PPase with the antibody since the

antibody did not show any cross-reactivity with H^+-PPase protein (data not shown). The two prominent bands on the silver-stained gel represent the heavy and light chain of the antibodies. Subunits of the H^+-ATPase as well as the H^+-PPase exhibited a higher staining intensity in preparations of SD plants than LD plants confirming the difference in the expression of two enzymes in plants cultivated under long or short photoperiods as reported before (Fischer-Schliebs *et al.* 1997).

Discussion

The H^+-PPase activated ATP-dependent H^+-transport was observed in *K. blossfeldiana* plants performing a low or high degree of CAM and confirmed data published by Marquardt-Jarczyk and Lüttge (1990) for the obligate CAM plant *K. daigremontiana*. The promotion of CAM expression in *K. blossfeldiana* cv. Tom Thumb by shortening the photoperiod is accompanied by an increased abundance of the vacuolar H^+-PPase and H^+-ATPase protein (Fischer-Schliebs *et al.* 1996) compared to plants of the same age cultivated continuously under LD conditions and performing only a weak CAM. A higher expression of the vacuolar H^+-ATPase in response to the transition of the metabolism to CAM can be explained by an increased requirement for energization the nocturnal accumulation of organic acids in the vacuoles of these plants. A direct role of the H^+-PPase in transtonoplast H^+-transport, at least in *K. daigremontiana* with its high nocturnal vacuolar malate acidification, appears unlikely for thermodynamic reasons. Therefore Marquardt-Jarczyk and Lüttge (1990), who observed the effect of H^+-PPase stimulated ATP-dependent H^+-transport for the first time, hypothesized that the H^+-PPase may participate in the kinetic regulation of the H^+-ATPase in CAM allowing adequate responses to changing requirements of cell metabolism, especially to vacuolar malate accumulation and remobilization. Although in *K. blossfeldiana* the expression of CAM as measured by the day-night changes of the malate concentration in the leaf cell sap was less dramatic in comparison to *K. daigremontiana* even after cultivation in SD conditions a stimulation of the ATP-dependent H^+-transport by a preceding activation of the H^+-PPase was observed. The relative stimulation was independent of the shift of the plant metabolism from weak to high CAM expression. Nevertheless, the absolute rates of the H^+-PPase-activated ATP-dependent H^+-transport of tonoplast vesicles of SD plants due to the higher abundance of the proteins in the vacuolar membrane were more than twofold higher than the rates of LD plants. The rates of H^+-PPase-stimulated ATP-dependent H^+-transport were also statistically significantly larger than the rates obtained with both substrates added simultaneously which was not the case in LD plants. Thus, a potentially higher capacity of the two H^+-translocating enzymes due to their co-ordinated action in energizing the vacuolar membrane in plants of *K. blossfeldiana* performing a high degree of CAM remains an intriguing possibility.

Recently an interaction of H^+-PPase and H^+-ATPase was also observed in tonoplast vesicles of *Acer pseudoplatanus* suspension cells (Fraichard *et al.* 1994). These authors found an inhibition of the PP_i-dependent H^+-transport when the

H^+-transport activity of the H^+-ATPase was blocked by inhibitors which did not affect the H^+-PPase itself. Since there is some evidence that the H^+-PPase rather acting merely as an H^+-translocating enzyme may catalyze both, H^+- and K^+-transport at the tonoplast (Davies *et al.* 1991), the authors argued that H^+/K^+-stoichiometry of the H^+-PPase could have been modified by the inhibition of the H^+-ATPase in favour of the transport of potassium. However, in *Kalanchoë* an involvement of potassium ions in the stimulation of the H^+-ATPase by the H^+-PPase was ruled out since the potassium forming ionophore valinomycin did not affect the H^+-PPase-activated ATP-dependent H^+-transport (Marquardt-Jarczyk and Lüttge 1990). Also other direct effects of PP_i on the H^+-ATPase (*e.g.* Jault *et al.* 1994) can be excluded since the PP_i-dependent stimulation of the H^+-ATPase is only observed under conditions when the H^+ PPase is active.

Support for the idea of a direct functional linkage of the two vacuolar H^+-translocating enzymes in *K. blossfeldiana* comes from the co-immunoprecipitation of considerable amounts of the H^+-PPase from solubilized tonoplast proteins of *K. blossfeldiana* with the H^+-ATPase when antibodies directed against the subunit A of the tonoplast H^+-ATPase of *M. crystallinum* were used. The close association of the H^+-PPase with the H^+ ATPase was found in tonoplast fractions of SD and LD plants of *K. blossfeldiana* and thus, it seems not to be correlated with the degree of CAM expression in these plants.

Interestingly, in another C_3-CAM intermediate plant, *M. crystallinum*, the H^+-PPase disappears during the induction of CAM by salt stress or plant ageing (Rockel *et al.* 1994). In C_3-plants where the H^+-PPase is present in high amounts in the vacuolar membrane a stimulation of the H^+-ATPase by the H^+-PPase was not observed (Marquardt-Jarczyk and Lüttge 1990). However, the basal ATP-dependent H^+-transport rates in tonoplast vesicles of *M. crystallinum* were already in the range of the high H^+-PP_iase-stimulated rates observed in *K. daigremontiana*. Hence, high rates of ATP-dependent H^+-transport are required in CAM but they may be established by different mechanisms, *i.e.*, by a co-operation of H^+-ATPase and H^+ PP_iase as in *Kalanchoë* or by changes in subunit composition of the H^+-ATPase as shown for *M. crystallinum* (Lüttge *et al.* 1995). Further research is required to resolve a possible regulatory interaction of the two H^+-translocating enzymes at molecular level in the tonoplast of different plant genera.

References

Ames, B.: Assay of inorganic phosphate, total phosphate and phosphatase. - Methods Enzymol. **8**: 115-118, 1966

Brulfert, J., Guerrier, D., Queiroz, O.: Photoperiodism and crassulacean acid metabolism. II. Relations between leaf aging and photoperiod in crassulacean acid metabolism induction. - Planta **154**: 332-338, 1982.

Davies, J.M., Rea, P.A., Sanders, D.: Vacuolar proton pumping pyrophosphatase in *Beta vulgaris* shows vectorial activation by potassium. - FEBS Lett. **278**: 66-68, 1991.

Fischer-Schliebs, E., Weber, P., Mariaux, J.-B., Ratajczak, R., Lüttge, U.: Response of the vacuolar H^+ PPase and H^+-ATPase of *Kalanchoë blossfeldiana* cv. Tom Thumb to the enhancement of CAM expression by short day treatment. - Planta, submitted, 1997.

Fraichard, A., Trossat, C., Magin, T., Pugin, A.: Influence of ATPase activity on PP_i dependent H^+-transport in tonoplast vesicles of *Acer pseudoplatanus*. - Plant Sci. **100**:129-138, 1994.

Hoffmann, B., Bentrup, F.: Two proton pumps operate in parallel across the tonoplast of vacuoles isolated from suspension cells of *Chenopodium rubrum* L. - Bot. Acta **102**: 297-301, 1989.

Hohorst, H.H.: L(-)-Malat, Bestimmung mit Malatdehydrogenase und NAD. - In: Bergmeyer, H.U. (ed.): Methoden der Enzymatischen Analyse. Vol. II. Pp. 1544-1548. Verlag Chemie, Weinheim 1970.

Jault, J.-M., Paik, S.R., Grodsky, N.B., Allison, W.: Lowered temperature or binding of pyrophosphate to sites for non-catalytic nucleotides modulate the ATPase activity of the beef heart mitochondrial F_1-ATPase by decreasing the affinity of a catalytic site for inhibitory MgADP. - Biochemistry **33**: 14979-14985, 1994.

Johannes, E., Felle, H.H.: Proton gradient across the tonoplast of *Riccia fluitans* as a result of the joint action of two electroenzymes. - Plant Physiol. **93**: 412-417, 1990.

Laemmli, U.K.: Cleavage of structural proteins of the bacteriophage T4. - Nature **227**: 680-685, 1970.

Leigh, R.A., Gordon-Weeks, R., Steele, S.H., Koren'kov, V.D.: The H^+-pumping inorganic pyrophosphatase of the vacuolar membrane of higher plants. - In: Blatt, M.R., Leigh, R.A., Sanders, D. (ed.): Membrane Transport in Plant and Fungi: Molecular Mechanisms and Control. Pp. 61-75. The Company of Biologists, Cambridge 1994.

Lüttge, U.: Carbon dioxide and water demand: Crassulacean acid metabolism (CAM), a versatile ecological adaption exemplifying the need for integration in ecophysiological work. - New Phytol. **106**: 593-629, 1987.

Lüttge, U., Smith, J.A.C., Marigo, G., Osmond, C.B.: Energetics of malate accumulation in the vacuoles of *Kalanchoë tubiflora* cells. - FEBS Lett. **126**: 81-84, 1981.

Lüttge, U., Ratajczak, R., Rausch, T., Rockel, B.: Stress responses of tonoplast proteins: an example for molecular ecophysiology and search for eco-enzymes. - Acta bot. neerl. **44**: 343-362, 1995.

Marquardt-Jarczyk, G., Lüttge, U.: PPase-activated ATP dependent H^+-transport at the tonoplast of mesophyll cells of the CAM plant *Kalanchoë daigremontiana*. - Bot. Acta **103**: 203-13, 1990.

Oaklay, B.R., Kirsch, D.B., Morris, N.R.: A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. - Anal. Biochem. **105**: 361-362, 1980.

Peterson, G.L.: A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. - Anal. Biochem. **83**: 346-356, 1977.

Ratajczak, R., Kemna, I., Lüttge, U.: Characteristics, partial purification and reconstitution of the vacuolar malate transporter of the CAM plant *Kalanchoe daigremontiana* Hamet et Perrier de la Bâthie. - Planta **195**: 226-236, 1994a.

Ratajczak, R., Richter, J., Lüttge, U.: Adaptation of the tonoplast V-type H^+-ATPase of *Mesembryanthemum crystallinum* to salt stress, C_3-CAM transition and plant age. - Plant Cell Environ. **17**: 1101-1112, 1994b.

Rea, P.A., Poole, R.J.: Vacuolar H^+-translocating pyrophosphatase. - Annu. Rev. Plant Physiol. Plant mol. Biol. **71**: 131-141, 1993.

Rockel, B., Ratajczak, R., Becker, A., Lüttge, U.: Changed densities and diameters of intramembrane tonoplast particles of *Mesembryanthemum crystallinum* in correlation with NaCl-induced CAM. - J. Plant Physiol. **143**: 318-324, 1994.

Wang, Y.Z., Leigh, R.A., Kaestner, K.H., Sze, H.: Electrogenic H^+ pumping pyrophosphatase in tonoplast vesicles of oat roots. - Plant Physiol. **81**: 497-502, 1986.