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Stress responses of tonoplast proteins: an example for molecular ecophysiology and the search for eco-enzymes*†

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SUMMARY

Molecular ecophysiology aims at understanding ecological adaptations at the level of molecules, and *vice versa*, the role of molecules in the ecological comportment of whole organisms. Hence, it continuously moves up and down a ladder of systems characterized by different levels of scaling like ecosystems and habitats, whole organisms, organs, tissues and cells, membranes and molecules.

Membranes with controlled transport mechanisms are essential for the separation from and contact with the environment. The vacuole of plant cells is an intermediary or permanent sink for solutes which are resources in metabolism or waste compounds.Therefore, the transport molecules of the tonoplast—the membrane separating the vacuole from the cytoplasm—play a key role in stress responses.

Among the membrane-transport enzymes of the tonoplast the H^+-pumping V_0V_1-ATPase has been characterized structurally and functionally, and it has recently been recognized as an enzyme both serving stress responses and undergoing stress-related modifications. Therefore, we call it an 'eco-enzyme'. We define an eco-enzyme as an enzyme which shows ecophysiological reactions by (i) mediating adaptations (i.e. in contrast to a house-keeping enzyme), and (ii) undergoing modification itself (i.e. in contrast to a stress enzyme).

The H^+-pumping tonoplast pyrophosphatase is known structurally, but its function in the whole plant remains enigmatic and therefore also its role in ecophysiology.

Secondary-active transporters are known to occur and to be essential in stress responses, but their molecular identity and therefore their precise role in molecular ecophysiology is as yet unknown.

Key-words: ATPase, eco-enzymes, plant stress, pyrophosphatase, tonoplast.

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*Dedicated to Professor Dr Johannes Willenbrink on the occasion of his 65th birthday.

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INTRODUCTION

Molecular ecophysiology

Ecophysiology considers problems at the level of ecosystems and habitats. Its aim is to trace the bases of ecological adaptation back to the biochemical and molecular levels and, *vice versa*, to start with molecules and evaluate the role of molecular responses in the performance of organisms in their habitat. Molecular phylogeny, i.e. tracing the degree of conservation or change of molecules at the nucleotide or protein level through evolution of organisms, is another face of this philosophy as it also, indirectly, addresses the role of environmental factors which impose selection pressure at the molecular level.

During the last years the perspectives of experimental plant ecophysiology have changed dramatically. Ecophysiological studies are now performed at different levels of organization spanning ecosystem studies and whole plant physiology to cells, membranes and molecules. There must be a continuous interchange in both directions moving up and down through these various levels of scaling. The tremendous progress made at the molecular level is above all the result of the introduction of new powerful molecular techniques. The refinement of robust albeit sensitive techniques in protein biochemistry, in combination with the polymerase chain reaction technology, allows us today to study evolutionarily conserved proteins of virtually any organism with unprecedented accuracy at the molecular level within a very short time. Previously existing barriers due to experimentally recalcitrant species have been overcome. In ecophysiology we may use protein and/or DNA sequence information obtained from a few model organisms to develop molecular probes for studying molecular adaptations to changing environments for almost any plant of interest.

In this context, the term molecular biology is commonly used for nucleic acid biochemistry. It must be noted, however, that the biology of molecules also comprises protein biochemistry. It is the aim of this review to demonstrate how real progress was obtained in ecophysiology only by combination of both experimental approaches. As a case story we chose protein molecules in a membrane, namely the tonoplast of higher plant cells. While numerous other examples could have been chosen for this purpose, what are the reasons to take a membrane and, in particular, the tonoplast?

Membranes

Membranes have been important at the onset of evolution. They allowed the early progenotes a 'material emancipation from the environment' (Netter 1959). They provide compartmentation as a basis of life. However, since only open systems with dynamic steady-state equilibria could lead the way to evolution of life, controlled permeability and regulated structures allowing traffic and exchange of solutes across the membranes also were essential right at the origin of life (Lüttge 1978). In extant organisms this regulated exchange is catalysed by proteins in the membranes that, as far as we know them to date, mostly prove to be highly conserved throughout phylogeny in their nucleotide-base and protein–amino-acid sequences.

The tonoplast

The tonoplast membrane in higher plant cells separates the central cell-sap vacuole from the cytoplasm. The vacuole is a reservoir serving as both a dump for waste and a reservoir of solutes which are resources for metabolism, and it is involved in regulation of water relations. Thus, evidently, traffic of solutes across the tonoplast must be of

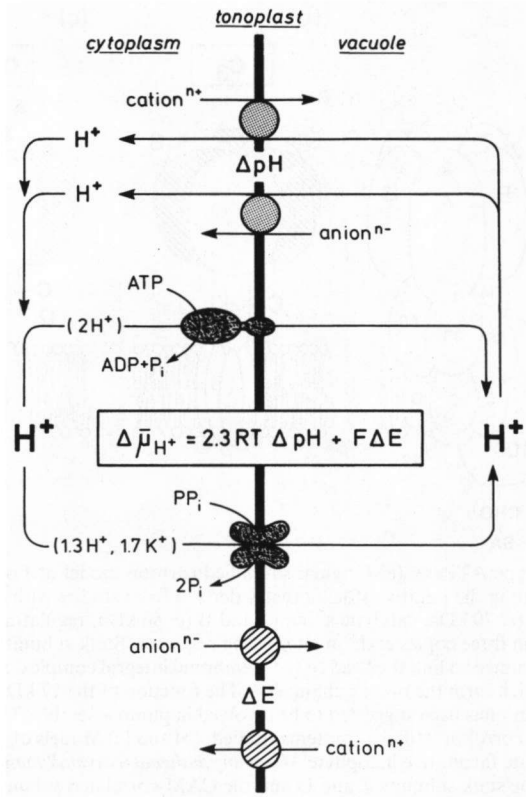

Fig. 1. Tonoplast proteins mediating primary- and secondary-active transport. The ATP- and inorganic pyrophosphate (PP_i)-consuming proton pumps transport protons into the vacuole (primary-active transport) and establish an H^+-electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) at the membrane, where R is the universal gas constant, T is temperature [K], ΔpH is the H^+-activity or pH gradient and ΔE is the electrical gradient at the membrane, and F is the Faraday. The PP_iase possibly may also pump K^+ ions at a ratio of 1·7 K^+/1·3 H^+ (see text). Symporters, antiporters and uniporters are driven by $\Delta\bar{\mu}_{H^+}$ (secondary-active transport). They transport H^+ ions and anions (or also electrically neutral solutes: not shown) in the same direction (symporters) or exchange protons and other cations (antiporters) or transport anions and cations alone (uniporters). Symporters and antiporters are largely driven by ΔpH and uniporters by ΔE.

prime importance in cellular stress reactions. The basic transport functions of the tonoplast are shown in Fig. 1. There are two systems of primary-active transport, namely the vacuolar of V-type ATPase and the tonoplast pyrophosphatase (tp-PP_iase) which pump protons into the vacuole using the free energy of the hydrolysis of ATP and inorganic pyrophosphate, respectively. These two membrane-transport enzymes are the major target of the review. They establish a proton electrochemical gradient at the tonoplast, which has two components, i.e. a gradient of proton activity (ΔpH) and a gradient of electrical potential (ΔE). These can energize movement of other solutes across the membrane in secondary-active transport via symporters or antiporters driven by ΔpH and uniporters driven by ΔE.

Sustaining all secondary-active transport mechanisms, the V-ATPase and the tp-PP_iase actively mediate stress responses. However, they may also themselves be subject to stress-evoked modification. Thus, especially for the V-ATPase, activity and regulation have very dynamic components not to be reconciled with the character of a

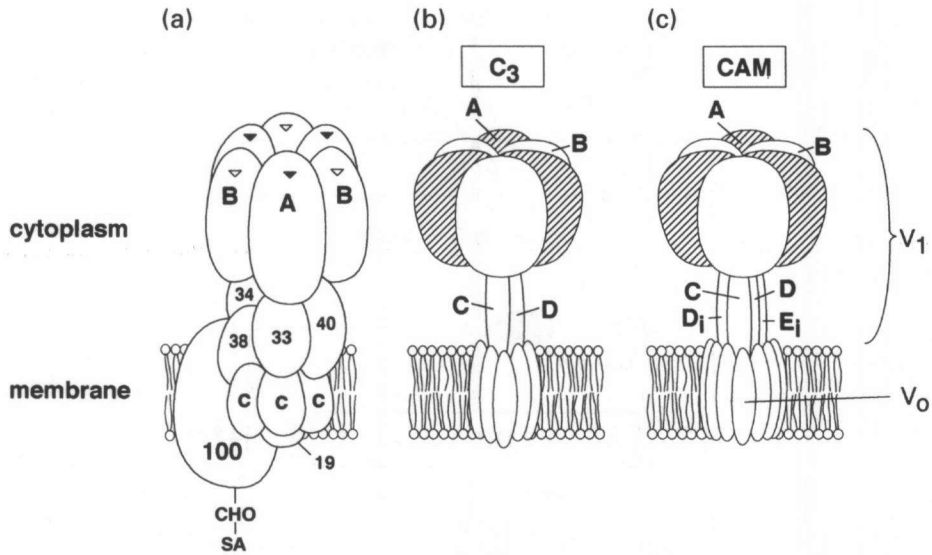

Fig. 2. Models of the V-type ATPase. (a) Original simplified cartoon model of Forgac (1992) of eukaryotic V-type H^+-ATPase indicating the putative stoichiometry derived from studies with clathrin coated vesicles of bovine brain. Subunits A (*c.* 70 kDa, catalytic subunit) and B (*c.* 60 kDa, regulatory subunit) form the head structure and are present in three copies each, in alternating sequence. Stalk subunits (33–40 kDa) are present in single copies and are assumed to link the head to the membrane integral complex. Subunit c, the proteolipid, is present in six copies, which form the proton channel(s). The function of the 19 kDa subunit is yet unknown, whereas the 100 kDa subunit has been suggested to be involved in pump assembly. The occurrence of the latter two subunits has not been corroborated in all systems studied. (b) and (c) Models of the tonoplast H^+-ATPase of the C_3/CAM intermediate facultative halophyte *Mesembryanthemum crystallinum* L. in the C_3-state (b) and the CAM-state (c), with the stalk subunits C and D and the CAM-correlated subunits D_i and E_i. The relative dimensions are based on measurements on electron-micrographs (I. Emig, unpublished). For details of subunit composition and stoichiometries see text.

typical housekeeping enzyme. Its complex ecophysiological reactions suggest that it is a true 'eco-enzyme' and an excellent example to elucidate molecular ecophysiology.

THE TONOPLAST H^+-ATPASE OR V-TYPE H^+-ATPASE

Subunit composition and three-dimensional structure: solving a plant's jigsaw puzzle with help from bovine brain and yeast

While the first report on the purification of a higher plant vacuolar H^+-ATPase (Mandala & Taiz 1986) indicated the presence of three major subunits, namely A (*c.* 70 kDa), B (*c.* 60 kDa), and c (*c.* 16 kDa), it became clear soon thereafter that the structure of this proton pump was more complex. Parallel investigations of the V-type H^+-ATPase subunit composition in different eukaryotic organisms including animals (Forgac 1989; Adachi *et al.* 1990; Arai *et al.* 1990; Zhang *et al.* 1992), fungi (Bowman *et al.* 1989; Kane *et al.* 1989) and plants (Bremberger *et al.* 1988; Parry *et al.* 1989; Matsuura-Endo *et al.* 1990; Ward & Sze 1992) revealed the high conservation of this proton pump among eukaryotes. Sodiumdodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified holoenzyme showed that a total of 8–10 subunits are forming the functional pump. Cartoon-type three-dimensional models have been proposed for the holoenzyme (Forgac 1989; Sze *et al.* 1992; Fig. 2): A peripheral

part, V_1, orientated towards the cytoplasm, and consisting of three copies each of the A and B subunits forming the ATPase head, with subunit A being the catalytic one, is attached via its stalk to the membrane integral part, V_0, which is possibly composed of different subunits including six copies of subunit c (the proteolipid) forming the proton channel.

The current models of the three-dimensional structure of the V-type H^+-ATPase holoenzyme reflect the outcome of biochemical dissociation and reconstitution studies as combined with electron microscopic examination. While chaotropic agents lead to dissociation of the V_1-part including all five subunits, a functional pump could be reconstituted from the V_1-stripped membranes and dialysed V_1 preparation (Puopolo & Forgac 1990). The reversible loss of the V_1-part from the membrane at low temperature *in vitro* as observed in the presence of MgATP (Moriyama & Nelson 1989) has been shown to be of physiological importance in living plant cells (Matsuura-Endo *et al.* 1992). The V-type H^+-ATPase from *Neurospora crassa* Shear and Dodge vacuoles was the first studied by electron microscopy (Bowman *et al.* 1989; Dschida & Bowman 1992). The pictures obtained indicated that indeed V_1 was fixed to the membrane integral V_0 part as a head and stalk-like structure. Soon after, these observations were confirmed for plant membrane preparations (Klink & Lüttge 1991; Taiz & Taiz 1991). Subsequently, dimensions of the various domains have been measured on electron micrographs of side views in negatively stained tonoplast vesicles (V_1-domain) and of membrane-integral particles on freeze-fracture replicas of tonoplast vesicles (I. Emig unpublished; Rockel *et al.* 1994) which made the cartoon more realistic (Fig. 2b and c, and see below). It is noteworthy that the structure of the V-type H^+-ATPase bears considerable resemblance to the F_0F_1-ATPases, i.e. ATP-synthesizing coupling factors, in accordance with their common evolutionary origin (Nelson & Taiz 1989; Kibak *et al.* 1992, see below).

Which subunit is essential and which is not: perspectives and limitations of yeast genetics. An enzyme of such high complexity made an unequivocal definition of its minimum subunit composition a formidable task. Although successful reconstitution of the solubilized plant enzyme provided some information about its structure, the combined efforts of yeast genetics and molecular biology eventually established the minimum subunit composition for a functional pump. Complementation studies with mutants having disrupted ATPase genes (Nelson & Nelson 1990; Foury 1990; Beltran *et al.* 1992; Manolson *et al.* 1992; Ho *et al.* 1993; Bachhawat *et al.* 1993) together with *in vitro* reconstitution experiments using recombinant subunit polypeptides (Peng *et al.* 1994) essentially confirmed the structural model shown in Fig. 2a. In yeast each subunit is encoded by a single gene, making it an ideal model system. However, although highly conserved with respect to many structural features the comparison of V-type H^+-ATPases from different eukaryotic organisms and even different organs of a single species revealed heterogeneity for some of its subunits. This is not surprising since this proton pump may reside on different endomembranes (Herman *et al.* 1994) and even on the plasma membrane in some animal cells (Forgac 1989; Hemken *et al.* 1992). Therefore, a functional specialization was to be expected. The first report on the cloning of a subunit B-isoform (Bernasconi *et al.* 1990) was soon followed by other reports on isoforms and gene families, respectively, for different subunits in both animal and plant species (Lai *et al.* 1991; Sze *et al.* 1992; Starke & Gogarten 1993).

While studying the specific role(s) of plant V-type H^+-ATPases in vacuolar ion and metabolite compartmentation in relation to environmental stresses (see below), we may certainly profit from new information on enzyme structure and regulation provided by yeast and other model organisms. Clearly, the recent report on the functional complementation of a yeast strain lacking subunit A with a plasmid encoded plant subunit A opens exciting perspectives for the study of structure–function relationships of the plant enzyme (Kim & Wilkins 1994). However, it is also evident that we are confronted with a quite different degree of complexity in space and time when studying higher plants with their different cell types and exposure to varying environmental conditions.

Sequence acquisition for higher plants: 'interkingdom jumping' by PCR technology

As only the use of homologous probes allows high sensitivity and specificity for gene expression studies, the polymerase chain reaction (PCR) was recently used to amplify partial cDNAs for subunits A, B and c from different plant species (Löw *et al.* 1995). The high conservation of protein sequences between different eukaryotic organisms for these V-type H^+-ATPase subunits (Kibak *et al.* 1992) allowed us to design consensus primers orientated against certain highly conserved sequence motifs for the amplification of the corresponding cDNA fragments (Fig. 3a). Because simple extraction of total RNA followed by first strand cDNA synthesis is all that is required for subsequent amplification by PCR, this experimental tool has helped to rapidly expand our knowledge on V-type H^+-ATPase gene expression in different plant species. As an example, Fig. 3b presents a dendrogram of a number of higher plant partial subunit c cDNA sequences as recently obtained by PCR using the same set of consensus primers for amplification. The PCR amplification was successful, even for the experimentally recalcitrant *Clusia minor* L. with its leather-like leaves and latex.

Responses to environmental stress: progress with molecular probes

Examples of secondary-active transport processes energized by the tonoplast H^+-ATPase, which are of special ecophysiological interest, are the transport of Cl^- through an anion channel and Na^+ *via* an Na^+/H^+-antiport into the vacuole under salinity stress conditions, and the nocturnal vacuolar accumulation of malic acid in CAM plants as an adaptation to drought stress (Fig. 4 and below).

Comparative studies with halophytes and non-halophytes investigating possible salinity responses of the tonoplast H^+-ATPase activity have been reviewed recently (Lüttge 1993). Stress-elicited increases in H^+-ATPase activity have been demonstrated. In suspension cells of *Nicotiana tabacum* L. adapted to 428 mM NaCl, which accumulate high concentrations of Na^+ and Cl^- and compartmentalize these ions in the vacuole, specific activities for ATP-hydrolysis and H^+-transport of the tonoplast H^+-ATPase were higher by factors of 4·0 and 2·9, respectively, than in unadapted cells (Reuveni *et al.* 1990) and kinetics changed from hyperbolic to sigmoidal after NaCl adaptation (Reuveni 1992).

The C_3-CAM intermediate halophyte *Mesembryanthemum crystallinum* L. has been particularly well studied in the authors' laboratories. In this species crassulaceen acid metabolism (CAM) is induced by salinity and drought stress as a special ecophysiological adaptation requiring massive transport of malic acid each night in addition to salt adaptation (Fig. 4; Lüttge 1993). The results of a concerted biochemical and molecular approach—in conjunction with the conceptional 'cross feeding' from yeast and bovine

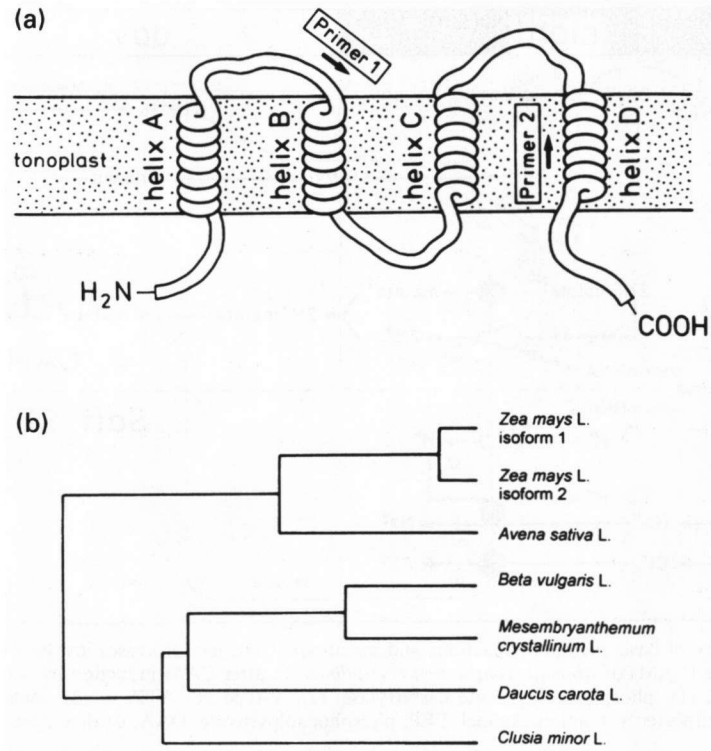

Fig. 3. Cartoon of subunit c with location of sequence motifs used for PCR primer design (a), and dendrogram of partial subunit c cDNA sequences from different plant species (b). Like subunits A and B (Kibak *et al.* 1992) subunit c is evolutionarily conserved among all eukaryotes. Using the same set of 'consensus' primers designed after conserved sequence motifs (corresponding to amino acids 49–56 and 133–140, respectively, of the *Avena sativa* L. sequence; Lai *et al.* 1991) partial cDNAs of 228 bp were amplified from first strand cDNA and sequenced. The monocotyledonous species are clearly separated from the dicotyledonous species. Isoforms are included for *Zea mays* L. Data from Löw *et al.* (1995) and unpublished work.

brain—strongly suggest that the proton pump at the tonoplast is not only actively involved in vacuolar salt compartmentation but is itself affected at the structural level.

In *M. crystallinum* the amount of tonoplast H^+-ATPase holoenzyme was assessed by radial immunodiffusion during the time course of an irrigation with 400 mM NaCl as compared to water (Ratajczak *et al.* 1994a). Initially, after the onset of salt treatment the amount of ATPase was increased reaching a maximum of 2·5-fold after 8 days. When salt stress was released the amount of ATPase declined immediately and reached the value found in control plants within a period of 2 days, indicating a substantial turnover of this proton pump.

The membrane-integral proteolipid (subunit-c). A time course study of transcript levels for subunits A, B and c indicated a rapid increase of all three messages in young leaves (about 2-fold after 8 hours), whereas in fully expanded leaves only mRNA for subunit c was increased after salt exposure (Löw *et al.* 1995). The apparent uncoupling of message levels for the different subunits in fully expanded leaves may indicate that the rate of *de novo* formation of the proton channel forming subunit-c may be a regulatory step for holoenzyme assembly and/or activity. Furthermore, silver-stained sodium

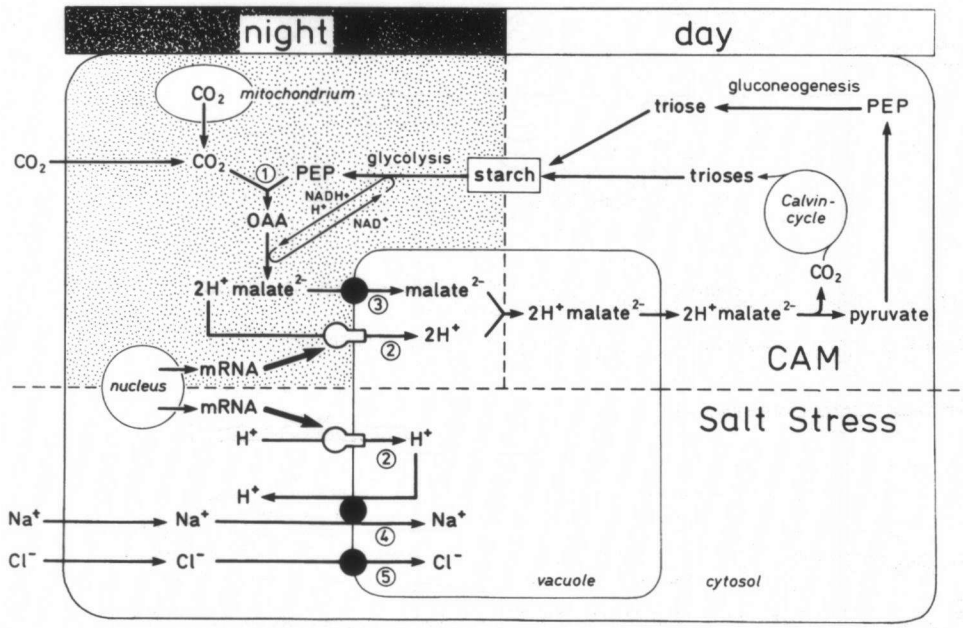

Fig. 4. Summary of basic metabolic reactions and membrane transport processes involved in crassulacean acid metabolism (CAM) of *Mesembryanthemum crystallinum* L. after CAM induction by NaCl. For further details see text. (1), phosphoenolpyruvate carboxylase, (2), V-type H^+-ATPase, (3), malate transporter, (4), Na^+/H^+ antiporter, (5), anion channel. PEP, phosphoenolpyruvate; OAA, oxaloacetate.

dodecylsulphate-polyacrylamide gels (SDS-PAGE) indicated an increase in the relative amount of subunit c protein during the time course of salt stress exposure (Rockel *et al.* 1994). It is as yet unclear whether the salt-induced increase of mRNAs coding for the tonoplast H^+-ATPase subunits A, B and c is the result of transcriptional activation or a changed message turnover. In fact, both possibilities have been recently shown to operate for different isoforms of the small subunit of RUBISCO in salt-stressed *M. crystallinum* (DeRocher & Bohnert 1993). A similar increase of subunit c mRNA and subunit c protein was recently found in fully expanded leaves of sugar beet (M. Kirsch, A. Zhigang & T. Rausch, unpublished). In *M. crystallinum* the diameter of intramembrane particles (IMPs), corresponding to the V_0 domain of the tonoplast H^+-ATPase, as assessed by freeze fracture analysis of tonoplast vesicles, increased from 7·3 nm to 9·1 nm (Rockel *et al.* 1994). Assuming V_0 is composed of several copies of subunit c (6 in the current model; Fig. 2a,b), this increase in IMP diameter could be due to an increase in the number of c subunits per H^+-ATPase holoenzyme (Fig. 2c). This may suggest a change in stoichiometry with an increased number of subunit-c copies per holoenzyme (Fig. 2b and c), which might be necessary for higher rates of proton transport activity, and/or improved attachment of the V_1-part (Ratajczak 1994; Lüttge *et al.* 1995).

In its natural habitat, drought-dependent CAM induction allows the annual species *M. crystallinum* to survive longer into the drought period and to bring a larger amount of seeds to maturity than other annuals (Winter *et al.* 1976, 1978). The change from C_3-photosynthesis to CAM can be monitored by recording gas exchange. The plants first develop a pattern of stomatal closure and suppressed gas exchange in the middle of the day (Fig. 5). It is shown by the observation of low CO_2-concentrations in the leaf air

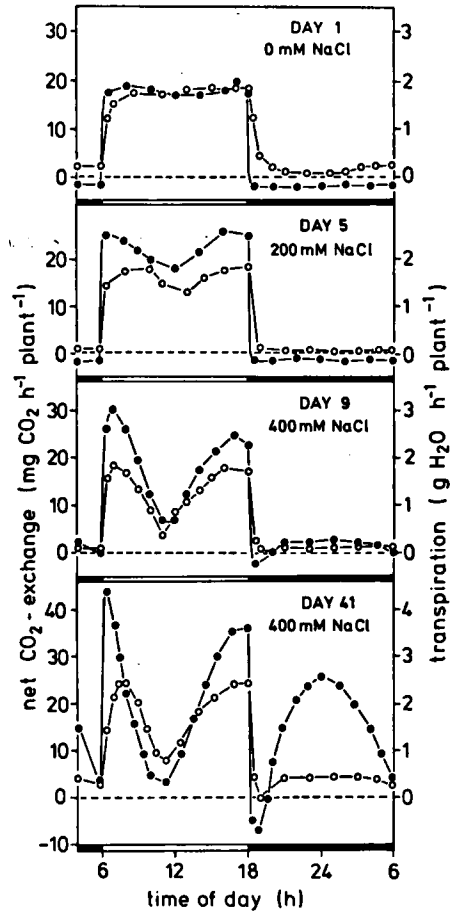

Fig. 5. Net CO_2-exchange (●) and transpiration (○) per whole plant of *Mesembryanthemum crystallinum* subjected to NaCl-stress: C_3-photosynthesis on day 0 (no stress); C_3-photosynthesis with midday-depression on day 5 after daily additions of 50 mM NaCl to the root medium had reached 200 mM; slight indication of nocturnal CO_2-uptake on day 9 at 400 mM NaCl; strong expression of CAM with the typical pattern of nocturnal CO_2-uptake, transitional peak of CO_2-uptake in the early morning, daytime stomatal closure and CO_2-uptake and assimilation via C_3-photosynthesis in the afternoon on day 41 at 400 mM NaCl. Positive values CO_2-uptake and H_2O-loss, negative values CO_2-loss. (From Winter & Lüttge 1979.)

spaces that this is like a typical midday depression of C_3-plants under drought stress (Winter & Gademann 1991). Only several days later, nocturnal CO_2-uptake associated with the vacuolar malic acid accumulation typical of CAM (Fig. 4) is elicited. The high internal CO_2-concentrations then observed during daytime stomatal closure show that this is no longer a C_3-midday depression but the daytime CAM-phase, during which vacuolar malic acid is remobilized and decarboxylated to develop CO_2 for assimilation behind closed stomata. Winter & Gademann (1991) have speculated that some kind of signal or message for the expression of CAM must be accumulated during the repeated C_3-midday depressions. In fact, analyses of mRNA for subunit-c of the ATPase not only show long-term increases within several days during the salt treatment, which are associated with increased protein levels, but also day/night oscillations without and immediately after the onset of the salt treatment, which are not associated with changes

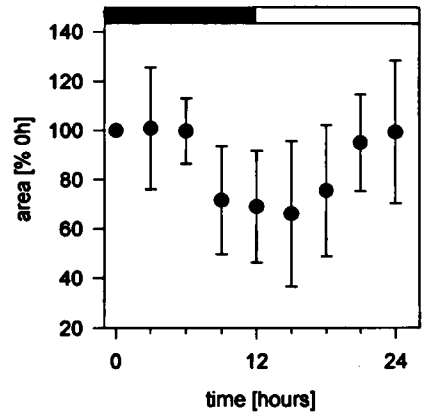

Fig. 6. Relative transcript levels for subunit c (16 kDa) of the tonoplast H^+-ATPase obtained by scanning of bands on Northern blots. The staining intensity at 0 h was arbitrarily set to 100%. Preparation of mRNA was performed from fully expanded leafs of well watered plants. The dark bar indicates the dark phase.

in protein levels (Löw *et al.* 1995; B. Rockel unpublished; Figs 6 and 7). The highest levels of subunit-c mRNA are found at the end of the light period when the plants have experienced transpirational stress, and the lowest levels are obtained at the end of the dark period when the plants could recover. Thus, stress and subunit-c transcription might well be related.

It remains to be seen if the message for synthesis of a subunit of an enzyme essential in the stress reactions, like the V-ATPase, is really involved in the salinity response and CAM induction time sequence. Nevertheless, the whole story already presents itself as an illustrative example of molecular ecophysiology. It has been worked out by a combination of observations in the field, gas exchange measurements, protein and nucleic acid biochemistry and electron microscopy, which may be summarized as follows:

1. germination of seeds and growth with C_3-photosynthesis of the annual species *M. crystallinum* after events of sufficient rainfall;
2. as drought sets in, daily periods of stress during midday;
3. increases and decreases in V-ATPase-subunit-c mRNA in a day/night fashion with peaks at the end of the day but without changes in corresponding protein levels;
4. increases in subunit-c mRNA, in corresponding protein levels and in diameters of intramembrane particles and expression of CAM as drought proceeds.

The importance of increased amounts of c-subunits is underlined by the finding that in another C_3-CAM intermediate species, *Kalanchoë blossfeldiana* cv. Tom Thumb (where CAM is induced by photoperiod (short days)), the relative staining intensity of subunit-c after SDS-PAGE of the immunoprecipitated holoenzyme (antiserum directed against subunit A) also showed an increase over other subunits after the metabolic switch from C_3-photosynthesis to CAM and that the diameter of the intra-membrane particles was also larger (Mariaux 1994). Again, this suggests different stoichiometries with a larger number of copies of subunit-c in the holoenzyme in the CAM-state.

Conversely, in barley (*Hordeum vulgare* L.) under salinity stress of 150–200 mM NaCl in the root medium there was only an increase in the amount of V-ATPase per unit of membrane area, as detected electron microscopically by immunogold labelling of negatively stained isolated tonoplast vesicles of the highly salt-tolerant cv. California

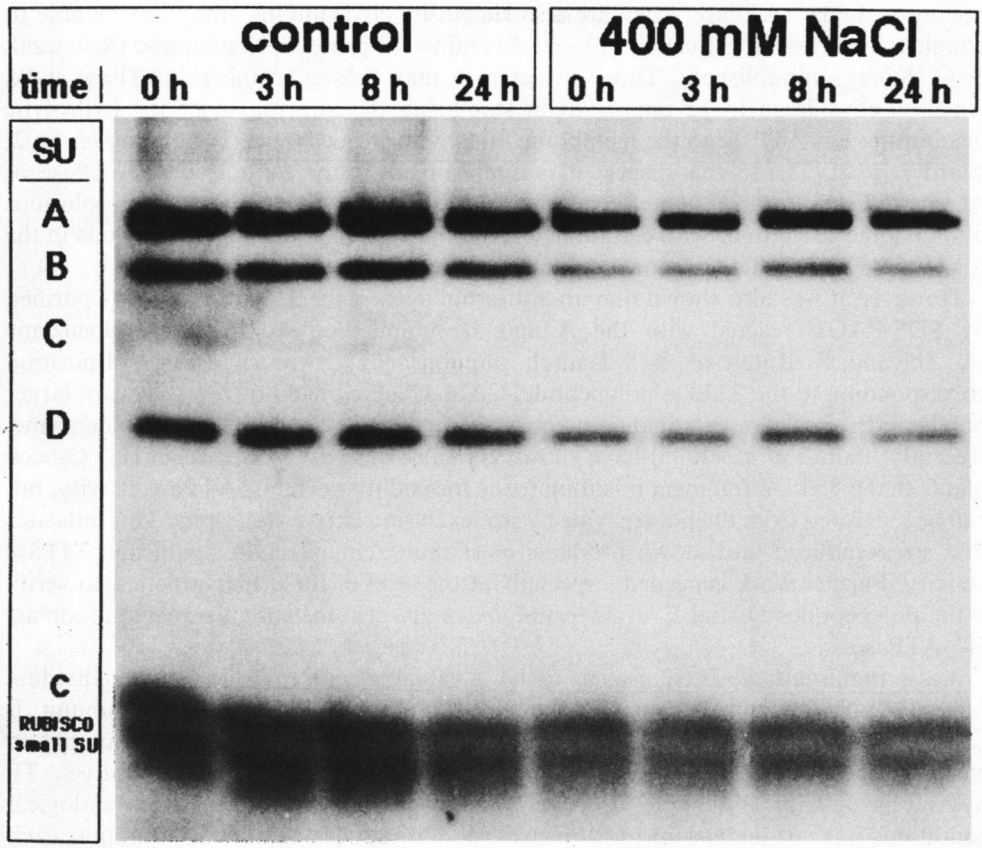

Fig. 7. Western blot analysis of protein in tonoplast vesicle preparations from control and salt-treated (400 mM NaCl) plants of *Mesembryanthemum crystallinum*. Immuno-staining was performed with an antiserum against the tonoplast H^+-ATPase holoenzyme of *Kalanchoë daigremontiana*. Tonoplast vesicles were isolated from fully expanded leaves at 0, 3, 8 and 24 h after the onset of salt treatment. Letters on the left-hand margin indicate the position of subunits of the tonoplast H^+-ATPase.

Mariout (Ratajczak *et al.* 1995) but no apparent change in subunit stoichiometries (Mariaux 1994).

The stalk domain (subunits C, D, E). Besides the increase of V_0, one of the most striking changes of the structure of the tonoplast H^+-ATPase of *M. crystallinum* at the protein level during salt treatment and the induction of CAM is the appearance of two tonoplast polypeptides which cross-react with an antiserum against the holoenzyme of the tonoplast H^+-ATPase of the obligate CAM plant *Kalanchoë daigremontiana* Hamet et Perrier de la Bâthie (Bremberger *et al.* 1988). These polypeptides with molecular masses of 32 (D_i) and 28 (E_i) kDa are strictly correlated with the expression of CAM and can be immunoprecipitated together with all other subunits of the tonoplast H^+-ATPase by an antiserum against subunit A, indicating that they are closely attached to the H^+-ATPase holoenzyme (Ratajczak *et al.* 1994a; Fig. 2c). The functions of the polypeptides D_i and E_i still are unclear. It could be shown that they do not affect the substrate affinity or inhibitor sensitivity of the tonoplast H^+-ATPase (Ratajczak 1994). With their molecular mass they are comparable with the subunits forming the stalk

region of the H^+-ATPase. They are also shown by cross-linking studies to be able to couple to head subunits (namely subunit A) and to the proteolipid subunit-c (Ratajczak & G. König, unpublished). Thus, in fact they may belong to the H^+-ATPase stalk, which is considered to be important in stabilizing the attachment of the hydrolytic head of the H^+-ATPase to the membrane integral proton channel (Puopolo *et al.* 1992; Ward *et al.* 1992). In this respect it is interesting that the tonoplast H^+-ATPase of *M. crystallinum* in the C_3-state seems to be more sensitive to incubation with solutions of high ionic strength or with certain detergents than the H^+-ATPase from plants in the CAM-state having the additional subunits (Berndt 1993).

However, it was also shown that an antiserum against the 32 kDa protein as purified by SDS-PAGE reacted with the A and B subunits of the tobacco holoenzyme (A. Zhigang, R. Ratajczak & T. Rausch, unpublished). Obviously, in the gel position corresponding to the 32 kDa polypeptide, CAM-specific breakdown products of larger polypeptides are present that remained attached to the V-ATPase holoenzyme. Recently, Bañuls *et al.* (1995) have shown for salt-stressed *Citrus sinensis* (L.) Osbeck plants that a 35 kDa fragment of subunit A is formed that exhibits ATPase activity, but can be separated from the holoenzyme by size-exclusion chromatography. This indicates that stress-induced breakdown products of subunit A may retain significant ATPase activity. Further work is needed, especially at the level of molecular genetics, to verify if the polypeptides D_i and E_i in *M. crystallinum* are genuine subunits of the tonoplast H^+-ATPase.

Yeast mutants have been proven to be a valuable tool to address the individual functions of different subunits of the H^+-ATPase holoenzyme. Thus, subunit E (26·6 kDa) seems to be essential for the tonoplast H^+-ATPase. Null *vam*4 mutants, in which the gene for subunit E was inactivated, exhibited no bafilomycin-sensitive ATP hydrolysis activity (Foury 1990). The mutants could survive under physiological conditions but not under temperature stress conditions. Using different mutants, each lacking a different subunit of the yeast vacuolar H^+-ATPase, Doherty & Kane (1993) showed that subunits A, B and E are essential for the assembly of V_1.

The ATPase head (subunits A, B). In salt-adapted suspension cultures of *N. tabacum* (see above) the steady-state level of mRNA for subunit A of the tonoplast H^+-ATPase was higher than in unadapted cells during the initial period of salt treatment (Narasimhan *et al.* 1991). Thus, transcription of the gene encoding subunit A and/or mRNA stability seem to be affected by short-term salt treatment.

Image analysis of electron micrographs of negatively stained V-type H^+-ATPase complexes of *M. crystallinum* reveals that the head is not always a hexamer as assumed from analogy with the F_0F_1-ATPase (see above and Fig. 2), but that a pentamer structure seems to be equally frequent (Kramer *et al.* 1995). Moreover, the head as a whole can readily be dissociated from the whole enzyme by chaotropic reagents (Lüttge *et al.* 1995). This indicates turnover of the V-type H^+-ATPase head, i.e. breakdown and reassembly as a whole, and/or of its individual subunits. The pentamers may represent intermediates of assembly and/or disassembly in accordance with the dynamic nature of tonoplast H^+-ATPase turnover.

The assembly of the bovine vacuolar H^+-ATPase was investigated at the protein level by immunoprecipitation of radiolabelled H^+-ATPase from different compartments of cells grown in monolayer culture (Myers & Forgac 1993). It could be shown that V_1 is assembled within 10–15 min. Within this period of time also intact V_0V_1 could be

detected. It was assumed by the authors that the assembly of V_0 and V_1 takes place in the endoplasmic reticulum prior to transport of the H^+-ATPase to the membranes of different cellular compartments. Interestingly, in the cytosolic fraction a population of assembled V_1 of unknown function was found.

Future perspectives for the molecular ecophysiology of the V-type H^+-ATPase

The elucidation of the dynamic nature of structural changes of the V-type H^+-ATPase in response to stress has confirmed our initial claim that only by a combination of protein biochemistry and molecular biology will a realistic picture emerge. In particular, we have observed that the stress response to salt exposure is organ-dependent and integrated into a developmental programme of differential gene expression. Our results have exposed several areas for future research. At the level of gene expression, a more refined analysis should address the important aspect of localized changes of V-type H^+-ATPase transcripts and protein at the tissue and cellular levels. Furthermore, the functional significance of the observed changes in gene expression should be tested with transgenic plants (although this is not an easy task for a multisubunit enzyme). At the protein level the dynamics of holoenzyme assembly and turnover in response to environmental factors should be investigated. Finally, the exact subunit stoichiometry, including potential changes after stress exposure, should be determined and related to structural stability and ATP/H^+-stoichiometry.

THE TONOPLAST H^+-PP_IASE

The H^+-transporting membrane PP_iase is exclusively found in tonoplasts of plants (tp-PP_iase) and is absent from other eukaryotes (Leigh *et al.* 1994). Hence, we cannot trace it through phylogeny like the V-type ATPase, although it is interesting to note that the tp-PP_iase has a consensus sequence in common with the proteolipid of the F_0F_1 and the V-ATPases (Nyren *et al.* 1993; Tanaka *et al.* 1993). The tp-PP_iase has been sequenced and cloned in *Arabidopsis thaliana* (L.) Heynh. and *Hordeum vulgare* L. (Sarafian *et al.* 1992; Tanaka *et al.* 1993). The molecular mass was found to be between 64 and 81 kDa in different materials, but various studies still leave it open whether the functional unit is the monomer, a dimer or even a tetramer of the same peptide (for review see Leigh *et al.* 1994). In any event, its fine structure is much simpler than that of the V-type ATPase with its several different subunits.

Functions in vitro *and* in vivo

Several functions of the tp-PP_iase have been shown *in vitro* using isolated vacuoles, tonoplast vesicles and reconstituted proteoliposomes (see Leigh *et al.* 1994 for review and additional citations given below), viz.

1. Hydrolysis of inorganic pyrophosphate.
2. H^+ transport into the vacuole against an H^+-electrochemical gradient.
3. K^+ transport into the vacuole (patch clamp studies by Davies *et al.* 1992).
4. PP_i-synthesis by reverse operation.
5. Kinetic stimulation of the V_0V_1-ATPase (Marquardt-Jarczyk & Lüttge 1990).

Nevertheless, open questions remain: What is the function of the tp-PP_iase *in vivo*? Why have two H^+-pumps with such different structures and properties evolved in the tonoplast of plants during phylogeny? Without knowing the function *in vivo* we cannot assess the role of this tonoplast enzyme in ecophysiology. Using our system of different

levels of scaling, in this case it really is the whole plant and the cell levels where information is needed to bridge the gap between ecology and molecular biology.

In the halophyte *Suaeda maritima* (L.) Dum. the tp-PP_iase did not show particular NaCl-stress-related activities and properties (Leach *et al.* 1990). In the facultative halophyte *M. crystallinum* the tp-PP_iase gradually disappeared in the leaves as plants aged under control conditions or under NaCl-salinity and in the state of CAM-expression (Bremberger & Lüttge 1992; Rockel *et al.* 1994). Thus, possibly the tp-PP_iase has little to do with salinity and nothing to do with CAM in *M. crystallinum*. Is the tp-PP_iase an essential enzyme of young growing cells? As in *M. crysallinum* and in other materials its activity was found to be particularly high in young organs. In growing tissues it may conserve the energy of inorganic pyrophosphate generated in synthetic pathways in the form of a proton electrochemical gradient at the tonoplast and thus, it may have functions in growth and turgor generation during cell extension via energizing secondary-active transporters (Chanson & Pilet 1987; Jelitto *et al.* 1992). Studies with transgenic plants, where the soluble cytosolic tp-PP_iase is overexpressed and PP_i-concentrations are much lower (Jelitto *et al.* 1992), as well as plants with reduced expression of the tp-PP_iase (Lerchl 1995) may soon cast more light on this issue.

Functions in stress responses?

In the genus of obligate and facultative CAM plants *Kalanchoë* the tp-PP_iase, in contrast to *M. crystallinum* (see above), may serve a function in the water-conserving mode of carbon acquisition CAM. It was shown for the obligate CAM plant *K. daigremontiana* that activation of the tp-PP_iase by its substrate $MgPP_i$ kinetically enhanced H^+-transport across the membrane of isolated tonoplast vesicles by the V-ATPase if the latter was activated by its substrate MgATP a few minutes later ($t_{\frac{1}{2}}$ 5–9 min). No other sequence of substrate additions led to a similar effect (Marquardt-Jarczyk & Lüttge 1990). The same result was obtained with *K. blossfeldiana* cv. Tom Thumb (E. Fischer-Schliebs, unpublished), where CAM is induced by short-day conditions and where the tp-PP_iase is much more strongly expressed in leaves in the CAM-state than in the C_3-state (Mariaux 1994).

For thermodynamic reasons it is unlikely that the tp-PP_iase is very important in driving nocturnal vacuolar malate accumulation in *Kalanchoë* by pumping protons into the vacuole because under cytoplasmic conditions, e.g. in cytoplasm isolated from sieve tubes, the free energy of PP_i-hydrolysis is likely to be only 19·3 kJ mol^{-1} (Geigenberger *et al.* 1993) while $\Delta\bar{\mu}_{H^+}$ at the tonoplast rises to 25 kJ mol^{-1} during the night (Lüttge *et al.* 1981). A K^+-transport function in CAM is also unlikely because malate accumulation is always stoichiometrically accompanied by accumulation of H^+ as counterion (Lüttge *et al.* 1975; Lüttge & Ball 1980). Moreover, in *Kalanchoë* the K^+ gradient is directed outwards from the vacuole requiring active transport in the opposite direction (Rona *et al.* 1980). However, by increasing the rate of V-ATPase driven H^+-pumping the tp-PP_iase in fact may support high rates of nocturnal malate accumulation during CAM and thus serve this adaptation to limited availability of water.

TRANSPORTERS

In contrast to the primary-active H^+-pumps, very little information is available for the structure of tonoplast-transport proteins serving secondary-active transport. This is due

to the low abundance of these symport, antiport and uniport carriers (Fig. 1) or ion-channels in the membrane. For example, in *K. daigremontiana* we estimate that the V-H^+-ATPase contributes about 36% to total tonoplast protein (Klink *et al.* 1990), while the putative malate carrier functioning in nocturnal accumulation of malate during CAM is probably less than 0·6% (Ratajczak *et al.* 1994b). All secondary-active tonoplast transporters are as yet only characterized kinetically, thermodynamically and by their transport physiology, i.e. by transport constants of carrier molecules and electrophysiological parameters obtained from patch-clamp studies of channels, by substrate specificities and pharmacological responses. In relation to stress responses at the tonoplast Cl^-, Na^+ and malate transporters would be of special interest (see above), but we know virtually nothing about the molecular nature of these transporters.

Channels for malate in the tonoplast of CAM plants have been functionally characterized (Iwasaki *et al.* 1992); however, purification and reconstitution experiments have shown that there should also be a malate carrier whose molecular mass must be in the range between 20 and 60 kDa (Ratajczak *et al.* 1994b; Lüttge *et al.* 1995).

An Na^+/H^+ antiporter which is highly inducible by NaCl-salinity has been detected in the tonoplast of various materials like barley, *Plantago* and sugar beet (Garbarino & DuPont 1988, 1989; Staal *et al.* 1991; Blumwald & Poole 1985, 1987). Biochemical studies using photolabelling of tonoplast from sugar beet cell suspensions with the inhibitor of vacuolar Na^+/H^+ exchange N-methyl-N-isobutyl-amiloride suggested that increased synthesis of a 170 kDa polypeptide was associated with increased Na^+/H^+ antiport capacity elicited by NaCl-stress (Barkla *et al.* 1990; Barkla & Blumwald 1991). However, no Na^+/H^+ antiporter has been cloned so far.

Hence, development in the field of secondary-active transporters is still very much behind that of the primary-active proton pumps and, as a consequence, their molecular ecophysiology still remains to be unravelled.

CONCLUSIONS AND EPILOGUE

Although much still needs to be learned about the detailed structure of the V-H^+-ATPase we know its general appearance, its evolution and its properties. We begin to unravel numerous environmental responses of this ATPase and recognize it as an eco-enzyme. Introducing this new term we define eco-enzyme as follows.

1. An enzyme which is directly involved in ecophysiological adaptations at the molecular level (in contrast to a house-keeping enzyme).
2. An enzyme which is post-translationally modified in response to stress (by (de)phosphorylation, oxidation/reduction, proteolysis, change in subunit stoichiometry).
3. An enzyme which shows a moderate change in gene expression at the transcript and/or protein level in response to stress (in contrast to strongly induced stress proteins).

All three criteria should be fulfilled. By introducing this new term we hope to fill the gap between two well-established categories, i.e. housekeeping enzymes and stress proteins. Clearly, the V-type H^+-ATPase, which has long been regarded as primarily serving housekeeping functions, has taught us a valuable lesson: essential adaptations to changing environment do not exclusively rely on massive *de novo* synthesis of specific stress proteins but may rather depend on more subtle changes in some of those enzymes, eco-enzymes, already present.

Table 1. Current state of knowledge (!) and open questions (?) regarding tonoplast proteins and their possible involvement in molecular ecophysiology

	Structure	Function *in planta*	Involvement in stress reactions
V_0V_1-H^+-ATPase	!?	!	!
TP-PP_iase	!	?	?
Transporters	?	!	!

The tp-PP_iase is simpler and consists of only one subunit, we already know more about its structure, albeit with some uncertainties whether a monomer, dimer or tetramer is the functional unit. However, there are still many doubts regarding its actual functions *in planta*.

Our present knowledge on the secondary-active transporters leaves no doubt that they are important participants in stress responses. However, we do not know their identity.

This marks the state of our knowledge regarding the possible involvement of tonoplast proteins in molecular ecophysiology and points out requirements for further work: ecophysiology of secondary active transporters awaits their identification; molecular ecophysiology of the tp-PP_iase awaits elucidation of its function(s) where currently studies with transgenic plants potentially lead the way; molecular ecophysiology of the V_0V_1-ATPase is well established (Table 1).

To the weary critic who considers the 'simplistic' molecular approach, here exemplified for tonoplast proteins, as being irrelevant for the understanding of 'real plants in a real environment' (Radin 1993), our answer is: Ultimately only the analysis of gene expression and function under conditions reflecting realistic environmental parameters will provide results which are relevant for the whole plant's performance. Therefore, molecular studies in ecophysiology should not be limited to plants raised in growth chambers or under glass-house conditions. General growth patterns in unrestricted soil space (root/shoot ratio) together with stochastically fluctuating environmental parameters (light, precipitation, temperature, wind, etc.) will profoundly modulate the comportment of the plant, which in turn will affect expression of stress-related genes. The ecophysiological significance of a molecular approach will ultimately depend on simultaneously monitoring gene expression *and* whole plant performance (photosynthetic activity, water relations) in the field. Thus, as ecophysiologists we have to learn how to move up and down in the scaling of nature.

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