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Significance of the V-type ATPase for the adaptation to stressful growth conditions and its regulation on the molecular and biochemical level

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Abstract

Two electrogenic H^+-pumps, the vacuolar type H^+-ATPase (V-ATPase) and the vacuolar pyrophosphatase, coexist at membranes of the secretory pathway of plants. The V-ATPase is the dominant H^+-pump at endomembranes of most plant cells, both in terms of protein amount and, frequently, also in activity. The V-ATPase is indispensable for plant growth under normal conditions due to its role in energizing secondary transport, maintenance of solute homeostasis and, possibly, in facilitating vesicle fusion. Under stress conditions such as salinity, drought, cold, acid stress, anoxia, and excess heavy metals in the soil, survival of the cells depends strongly on maintaining or adjusting the activity of the V-ATPase. Regulation of gene expression and activity are involved in adapting the V-ATPase on long- and short-term bases. The mechanisms known to regulate the V-ATPase are summarized in this paper with an emphasis on their implications for growth and development under stress.

Key words: V-ATPase, electrogenic pump, regulation of gene expression, stress conditions.

Introduction

Eukaryotic cells contain extensive internal membranes which separate the plasmatic from extraplasmatic compartments. The organelles of the secretory pathway, including the endoplasmatic reticulum, Golgi, lysosomes, and the vacuole, are linked by intracellular vesicle trafficking which also involves the plasma membrane. In plants, particularly due to the size of the vacuole, the secretory organelles account for the majority of the cell volume. These organelles, including the machinery of secretory vesicular transport, play a crucial role in plant responses to various stress regimes. Two of the most important responses are that (1) the composition of the secretory organelles and limiting membranes must be adapted to the needs for survival and development under the prevailing growth condition, for example, to low or high temperature, and (2) various solutes are transported into and mobilized from secretory compartments as an adaptive mechanism under stress. Additionally, secretory compartments are suggested to serve as sensor sites to determine stress levels, and to trigger downstream events which allow for the physiological adaptation to the stressor.

A small set of membrane proteins, directly energized through the hydrolysis of MgATP, MgGTP or $MgPP_i$, constitutes the basic framework for establishing distinct chemical milieus in the plasmatic and extraplasmatic

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Abbreviations: ABC transporters, ATP binding cassette type transporters; atpv, subunit of the vacuolar ATPase; IMP, intramembrane particles; PMF, proton motive force.

compartments. Among these are the Ca^{2+}-ATPase, the V-type H^+-ATPase and an as yet unknown number of solute transporters of the ATP binding cassette type superfamily (ABC transporters: Rea *et al*., 1998; Theodoulou, 2000). Insights into the genetic complexity of ABC transporters in plants, their substrates, role in homeostasis, compartmentation, and stress response has been only slowly emerging. It was not until 1993, that Martinoia and coworkers characterized the ATP-dependent glutathione-*S*-conjugate export pump of barley (Martinoia *et al*., 1993). Conversely, the vacuolar H^+-ATPase has been studied for approximately 25 years since its initial identification as the major NO_3^--sensitive ATP hydrolysing activity of the vacuolar membrane. During this period, a large body of information on the structure, function and regulation of the V-ATPase of plants has also been accumulated. At the end of 2000, for the first time sequence information became available for all subunits of the V-ATPase, for two plant species: *Arabidopsis thaliana* and *Mesembryanthemum crystallinum* (Table 1). Employing this additional information, it will be possible to elucidate new features of the V-ATPase. The topic of this review will be the predominant role that the V-ATPase plays in stress responses.

Energization of endomembranes by the V-type H^+-ATPase

Two H^+-pumps energize plant endomembranes: the V-ATPase and the H^+-pyrophosphatase. Both are electrogenic and create a PMF for secondary active transport (Davies, 1997). The H^+-pyrophosphatase has a simple homodimeric structure of two 71–80 kDa subunits. Its function is seen in (1) alternative or additional energization of the tonoplast in case of insufficient V-ATPase activity, (2) a role in pyrophosphate metabolism and (3) in K^+ homeostasis (for a review see Davies, 1997; Baltscheffsky *et al*., 1999; Maeshima, 2000). However, a direct function in K^+-transport, as was previously concluded from patch clamp studies, could not be supported in reconstitution experiments (Maeshima, 2000). In discussing the features of the V-ATPase, the presence of the H^+-pyrophosphatase at the tonoplast has to be kept in mind at all times, because of its possible

Table 1. *Compilation of genes (ORFs) in the genome of* Arabidopsis thaliana *which code for putative subunits of the V-ATPase*

The table gives the location on the chromosome, the predicted size of the gene product and a comparison of sequence similarity at the amino acid level between the respective deduced *Arabidopsis thaliana* proteins and the cloned sequences of *Mesembryanthemum crystallinum* (C Kluge, N Tavakoli, D Golldack, KJ Dietz, unpublished results).

Subunit of the vacuolar ATPase	Protein entry code(s) in the *Arabidopsis* genome project	Location on chromsome	Predicted size of the ORF/molecular mass	Sequence identity compared to *M. crystallinum* on the amino acid level
A: AtVHA-A	At1g78900	1	623 aa/ 68.8 kDa	91%
B: AtVHA-B	At1g76030	1	486 aa/ 54.1 kDa	94%
	At4g38510	4	487 aa/ 54.3 kDa	96%
	At1g20260	1	468 aa/ 52.1 kDa	95%
C: AtVHA-C	At1g12840	1	370 aa/ 42.0 kDa	76%
D: AtVHA-D	At3g58730	3	261 aa/ 29.0 kDa	75% (EST)
E: AtVHA-E	At1g64200	1	237 aa/ 27.1 kDa	76%
	At4g11150	4	230 aa/ 26.1 kDa	76%
	At3g08560	3	235 aa/ 26.8 kDa	66%
F: AtVHA-F	At4g02620	4	128 aa/ 14.3 kDa	83%
G: AtVHA-G	At4g23710	4	106 aa/ 11.8 kDa	54%
	At3g01390	3	110 aa/ 12.4 kDa	59%
H: AtVHA-H	At3g42050	3	441 aa/ 50.3 kDa	70%
a: AtVHA-a	At2g21410	2	821 aa/ 93.1 kDa	71%
	At4g39080	4	843 aa/ 95.2 kDa	72%
	At2g28520	2	780 aa/ 89.3 kDa	70%
d: AtVHA-d	At3g28710	3	351 aa/ 40.8 kDa	94% (EST)
	At3g28715	3	343 aa/ 39.8 kDa	94% (EST)
c: AtVHA-c	At1g19910	1	165 aa/ 16.7 kDa	96%
	At4g38920	4	164 aa/ 16.6 kDa	96%
	At4g34720	4	164 aa/ 16.6 kDa	96%
	At2g16510	2	164 aa/ 16.6 kDa	96%
	At1g75630	1	166 aa/ 16.7 kDa	96%
c′: AtVHA-c′	no sequence information			
c″: AtVHA-c″	At4g32530	4	180 aa/ 18.4 kDa	
	At2g25610	2	178 aa/ 18.2 kDa	
e: AtVHA-e	At4g26710	4	70 aa/ 7.6 kDa	80% (EST)
	At8g55296	5	70 aa/ 7.7 kDa	80% (EST)

interference with structure and function of V-ATPase (Fischer-Schliebs *et al.*, 1997).

Structure of the V-type H^+-ATPase

The V-ATPase is a multiheteromeric complex of at least 11 different subunits which are arranged in a head/stalk/base arrangement (Fig. 1A). The molecular mass of the whole complex is over 700 kDa. Of the 11 distinct subunits (i.e. atpv A through to atpv H, atpv a, c and d) only three have an unequivocally assigned position in the holoenzyme complex. In an alternating arrangement, three copies each of atpv A and B form the head structure which extends into the cytoplasm (V1-sector), while six (or more) copies of the atpv c form the base (V0-sector), which is a doughnut-shaped ring in the tonoplast. The suggestions on the structural arrangement of the subunits have been refined based mainly on work with V-ATPase of yeast, as well as that with the midgut of the larval tobacco hornworm *Manduca sexta*, and the, although low, homology with subunits of the F-ATP synthase of plastids and mitochondria. Despite all progress, the fine structure of V-ATPase is still subject to speculation. An example of a controversial hypothesis on V-ATPase structure is the search for the polypeptide which is homologous to the γ-subunit of the F-ATP synthase, and transduces the conformational change in the head to the proton-conducting membrane base. Based

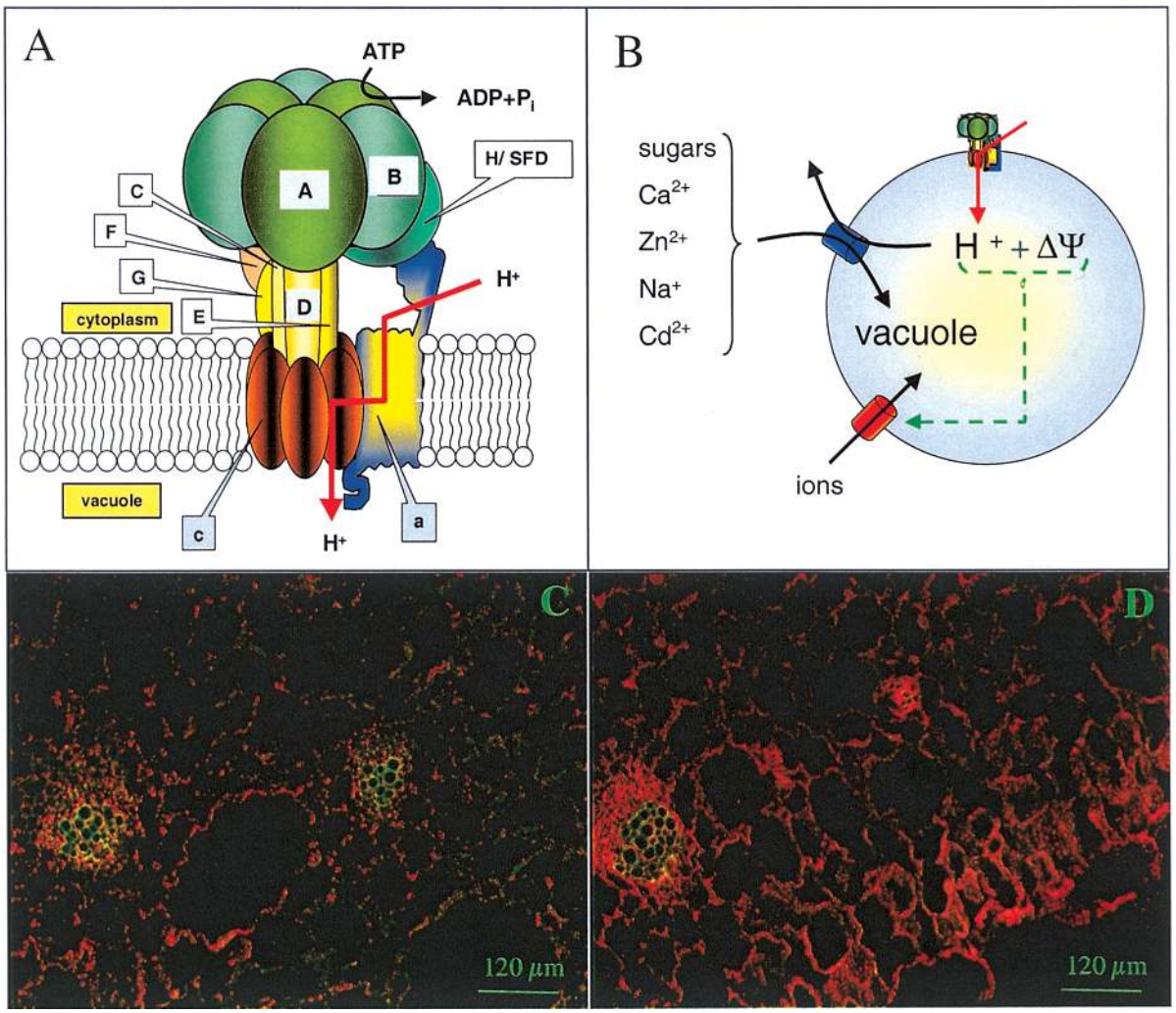

Fig. 1. Structure, basic functions and cell-specific expression of V-ATPase. (A) Hypothetical arrangement of atpv A-H, a and c in the holocomplex of the functional V-ATPase. Subunits coloured in brown or yellow constitute the proposed rotor structure, subunits in green and blue the stator. (B) Function of the V-ATPase in energizing the tonoplast or other membranes of the secretory pathway. The proton motive force built up by primary electrogenic pumps consists of the ΔpH and a small membrane potential ΔΨ and drives H^+-antiporters which carry ions such as Na and Zn (blue transporter). Uniporters and channels (red) transport other substrates which may accumulate following protonation (acid trap) or are distributed according to ΔΨ. (C, D) Immunolocalization of V-ATPase subunit E in leaf cross-sections of 10-week-old *Mesembryanthemum crystallinum*.(C) Control plant. (D) Plant stressed with 400 mM NaCl for 72 h. Subunit E localization is shown with red fluorescence signals.

on structural predictions, Nelson *et al*. suggested that atpv D of V-ATPase is structurally equivalent to subunit γ (Nelson *et al*., 1995). On the other hand, Grüber *et al*. concluded from trypsination and dissociation experiments with isolated *Manduca* V-ATPase that atpv E rather than atpv D was the most likely structural and functional homologue of the γ-subunit (Grüber *et al*., 2000). For a more detailed description of V-ATPase structure and function the reader is referred to a recent review (Ratajczak, 2000).

Catalysis and dynamics

V-ATPase and F-ATP synthase are related enzymes, and are assumed to have evolved from a common ancestor (Nelson *et al*., 1992). The F-ATPase is frequently used as a reference enzyme, whose properties can be extrapolated to the V-ATPase. Although there are differences between both types of ATPases in structure, activity regulation and inhibitor characteristics, it should be noted, however, that there are similarities in molecular structure on the nm-scale and a (very) low level of sequence conservation of atpv A, B and c. In the F-ATP synthase, the suggested rotary mechanism of catalysis couples proton conduction in the V0-sector to ATP synthesis in the V1-head (McCarty *et al*., 2000). This model assumes a rotor-like structure, composed of atp c and stalk subunits, which rotates against the stator, built from membrane bound subunits, other peripheral stalk subunits and the head structure of atp A and atp B. Each step of catalysis corresponds to a partial turn of the rotor by 120° in accord with the three-fold symmetry of the head. Applying this model to the V-ATPase, successive hydrolysis of ATP by the three catalytic subunits atpv A in V1 would complete the 360° rotation of the rotor. The H^+/ATP stochiometry would then depend on the number of proton binding sites in each 120° sector of V0 which are vectorially unloaded. Assuming one H^+-binding site at each atp c-subunit, and two atpv c copies per 120° sector, the maximum stochiometry would be 2 H^+/ATP, with two binding sites per atpv c the value would increase to 4 H^+/ATP. A value of 2 was frequently measured, but values ranged from 1.7–3.3 H^+/ATP in one study, depending on the transtonoplast pH gradient (Davies *et al*., 1994; for review see Ratajczak, 2000). The atpv c 16 kDa subunits arrange ring-like in the tonoplast, and this structure can be visualized as intramembrane particles (IMP) on freeze-fractures of tonoplast vesicles. Interestingly, the IMP diameter increased in *Kalanchoë blossfeldiana* Poellnitz cv. Tom Thumb plants with strong CAM expression as compared to control plants with low CAM expression (Mariaux *et al*., 1998). This may indicate an increased number of atpv c-subunits in the rotor and represent the structural basis for an H^+/ATP stochiometry higher than 2 even under the assumption of only one H^+-binding site per one atpv c-molecule. From considerations of the energetics, a vacuolar H^+-accumulation to pH 3 or lower depends on a H^+/ATP-ratio of <2 (Davies *et al*., 1994).

Distribution of vacuolar type H^+-ATPase in plant cells

Immunochemical and biochemical studies have revealed that the V-ATPase is not localized exclusively to the tonoplast of mature vacuoles, but is also associated with ER, provacuoles, plasma membrane, and other membranes of the secretory pathway (Herman *et al*., 1994; Robinson *et al*., 1996). At present the precise function of V-ATPases at these membranes, particularly at the plasma membrane is subject to speculation.

H^+-ATPase and stress

In the light of the basic function in endomembrane energization and compartmentation, a principal role of the V-ATPase during development and stress adaptation of plants has to be expected, and has been demonstrated, with salinity being the best studied example.

Salinity

In plants, the central vacuole plays an important role for the regulation of cytoplasmic ion homeostasis. Efficient exclusion of excess sodium from the cytoplasmic compartment and the vacuolar sodium accumulation are the main mechanisms for the adaptation of plants to excess sodium chloride concentrations. The vacuolar sodium sequestration is mediated by a secondary active Na^+/H^+-antiport at the tonoplast (Barkla *et al*., 1995; Apse *et al*., 1999) and it is energized by a proton motive force that is driven by the vacuolar H^+-ATPase (Fig. 1B).

Detailed analyses of the regulation of the V-ATPase activity and of the transcription and translation of V-ATPase subunits in response to salinity stress have been carried out for the facultative halophyte *Mesembryanthemum crystallinum* (common ice plant). It has been shown that the activity of the V-ATPase increases in *M. crystallinum* under treatment with NaCl (Ratajczak *et al*., 1994; Tsiantis *et al*., 1996). In contrast, the activity of the second vacuolar H^+ pump, the vacuolar H^+-transporting pyrophosphatase, decreased (Bremberger *et al*., 1988).

Transcriptional activation of the V-ATPase subunit c has been shown in leaves and roots of 6-week-old *M. crystallinum* treated with 350 mM NaCl for 24 h (Tsiantis *et al*., 1996). An increase of subunit c mRNA levels but not of subunits A and B was observed in fully expanded leaves of 4-week-old *M. crystallinum* after 8 h treatment with 400 mM NaCl (Löw *et al*., 1996). These data indicate non-co-ordinated regulation of the transcription of V-ATPase subunits as an early salinity

stress response in the ice plant. In contrast, in roots and young leaves transcription of subunit c as well as subunits A and B was enhanced. In leaf tissue of salt-adapted *M. crystallinum* at the age of 5 and 10 weeks, co-ordinated transcriptional activation of the V-ATPase subunits A, B, E, F and c (Golldack and Dietz, 2000) as well as subunit G (D Golldack and KJ Dietz, unpublished results) occurs. According to these data, the non-co-ordinated regulation of expression of V-ATPase subunits demonstrated previously (Löw *et al*., 1996) for *M. crystallinum* is an early stress effect and might be due to different turnover rates of the subunits or due to different signalling pathways regulating the transcription of the subunits. In *M. crystallinum* adapted to high salinity the transcript amounts of different subunits increased in comparison to non-stressed control plants in a co-ordinated way indicating increased amounts of the V-ATPase holoenzyme complex. These data correspond with results from physiological studies that demonstrated increased activity of the V-ATPase in salt-adapted *M. crystallinum* (Ratajczak *et al*., 1994; Barkla *et al*., 1995).

In the ice plant the expression of the V-ATPase in response to salinity stress is differentially regulated in specific tissues and cells. With *in situ* hybridization and histochemical detection, transcripts and protein of the V-ATPase subunit E were detected in all cell types in root and leaf tissue. During salinity stress, a decline in the expression of subunit E was found in root cortex cells and the root vascular cylinder whereas in leaves an increase was found with the strongest signals surrounding the vasculature (Golldack and Dietz, 2000) (Fig. 1C, D). These cell-specific transcript and protein levels are likely to reflect a unique involvement of the various cell types and tissues in sodium transport and accumulation and hence of the V-ATPase within the plant.

Increases of the transcript levels of the subunit A of the V-ATPase are also known from salt-stressed and salt-adapted cell suspension cultures of tobacco (Narasimhan *et al*., 1991). In the halotolerant sugar beet (*Beta vulgaris* L.), as well as in *B. vulgaris* suspension culture cells, expression of the subunits A and c of the V-ATPase were stimulated by NaCl in a co-ordinated way (Kirsch *et al*., 1996; Lehr *et al*., 1999). In addition, in salt-treated sugar beet suspension cells, the promoter activity of subunits A and c, measured as the expression of the luciferase reporter gene, increased under salt stress (Lehr *et al*., 1999).

In contrast to the enhanced gene expression of the vacuolar ATPase in salt-treated halotolerant plants, many glycophytes do not show salt-dependent regulation of the enzyme. In tomato, the subunit A mRNA levels increased transiently under salt stress but showed control levels after 3 d of further treatment (Binzel, 1995). In barley plants exposed to 300 mM NaCl subunit E proteins showed a slight increase in the root tissue but no change in leaves (Dietz *et al*., 1995). In *Arabidopsis thaliana*, the expression of subunit D of the vacuolar ATPase was not modified by NaCl treatment (Kluge *et al*., 1999). Interestingly, seedlings of *M. crystallinum* that are not salt-tolerant do not show transcriptional changes of the V-ATPase subunits A, B, E, F, and c under salt stress (Golldack and Dietz, 2000). These data indicate that the ability to respond to salinity stress with changes in the gene expression of the vacuolar ATPase might be a prerequisite and a characteristic of salt tolerance in plants. A drawback is that many of these studies either deal with mRNA or protein levels, and rarely were attempts made to measure V-ATPase activity. However, all these parameters are important when assessing the function of the V-ATPase under stress (cf. Ratajczak *et al*., 1994; Ratajczak, 2000). A promising approach for future studies on the physiological role of the V-ATPase in stress resistance will be the characterization of mutant plants as, for example, T-DNA-insertion mutants carrying insertions or deletions in selected subunits of the enzyme complex.

Drought

Little attention has been paid to the function of the V-ATPase under drought. Since maintenance of vacuolar compartmentation is of fundamental importance particularly under conditions of water and turgor loss and increasing ion and metabolite concentrations, there is a need to investigate in more detail the expression and activity of V-ATPase under water stress. It has been shown that the message level of atpv A did not increase in tomato under drought stress (Binzel and Dunlap, 1995). The authors concluded that abscisic acid is not involved in upregulation of ATPase under salinity, since abscisic acid levels increased similarly under both conditions, drought and NaCl-stress. Conversely, an increased V-ATPase activity was observed following the application of abscisic acid to barley (Kasai *et al*., 1993), and in *M. crystallinum* transcriptional activation of the V-ATPase subunit c could be induced in leaf tissue by ABA application to the leaves (Tsiantis *et al*., 1996). Obviously, the response of V-ATPase gene expression and activity under drought and also in response to stress-induced elevated or exogenously applied abscisic acid needs to be investigated in more detail if a clear conclusion on the relationship between V-ATPase and drought is to be drawn.

Heavy metals

Although a lot of cellular mechanisms, such as the synthesis of phytochelatins and metallothioneins, are generally believed to be involved in the detoxification of heavy metals, a very important role is reserved for transmembrane transport of these toxicants. Differential

resistance of a single species such as barley to various heavy metals involves distinct capacities for vacuolar compartmentation (Brune *et al*., 1995). Metal tolerance versus metal sensitivity of closely related species or genotypes also appears to depend on additional membrane, including tonoplast, transporters (Verkleij *et al*., 1998; Chardonnens *et al*., 1999). Therefore, it is reasonable to assume that the V-ATPase is affected under conditions of heavy metal exposure. Little information is available on the influence of heavy metals on either the structure or the activity of the V-ATPase. However, there is some experimental data on the effect of V-ATPase activity on metal transport. Although all heavy metals are toxic when present in excess, plants have a need for essential metals, such as Fe, Zn and Ni. Based on research on yeast metal transporters, it is assumed that any particular metal has both a high- and a low-affinity uptake system, the use of which depends on the abundance of the metal (Guerinot, 2000). Additionally, intracellular transport systems need to be present. This makes the understanding of membrane transport processes involved in metal ion homeostasis a complicated matter. However, the situation becomes even more difficult when dealing with plants that have adapted to high metal concentrations in the environment, since these may have evolved cellular tolerance mechanisms which involve vacuolar transporters (Chardonnens *et al.*, 1999).

In dealing with metal transport across membranes, it is of importance to distinguish between 'household' transport and tolerance mechanisms, since these may depend on different proteins, which could compete for substrates and interfere with each other's activity. Even more important is to keep in mind that, although a certain substrate may be transported in *in vitro* studies, it may not necessarily be transported *in vivo*. As was stated earlier (Williams *et al*., 2000), it is of the utmost importance to ascertain the concentrations of metal ions the transporters encounter in the plant prior to drawing conclusions about the transporter substrate.

Based on such considerations, one might look critically at proton gradient-dependent transport of metals across the tonoplast. This type of antiporter activity depends on the presence of a proton gradient across the vacuolar membrane and thus, indirectly, on the V-ATPase. Although metal-proton antiport activity has been reported by several authors for several metals, i.e. Cd, Zn, and Mn in oat roots (Salt and Wagner, 1993; Gonzales *et al*., 1999) and for Zn in *Silene vulgaris* (Chardonnens *et al*., 1999), other authors could not detect such activity for Cd, either in oat or *Silene* (Schumaker and Sze, 1986; AN Chardonnens and PLM Koevoets, unpublished results). Moreover, it was found that under circumstances favouring antiport activity, Ni associates with the tonoplast of oat roots, rather than with the vacuolar lumen (Gries and Wagner, 1998).

Clemens *et al*. suggested that non-essential metal ions, such as Cd, are most likely transported across plant membranes via transporters for essential cations (Clemens *et al*., 1998). These authors describe a Ca transporter from the wheat plasma membrane which mediates Cd transport in yeast, and is blocked by Ca. Gonzales *et al*., however, reported that Cd and Ca transport activity across the oat root tonoplast, presumably by antiport activity, showed different sensitivities to various inhibitors known to affect Ca transport, which might suggest that these ions do not make use of the same carrier (Gonzales *et al*., 1999). However, the mode of action of inhibitors is largely unknown, and may account for different effects on different substrates.

Antiport activity across the tonoplast requires a proton gradient, and is thus largely dependent on the activity of the V-ATPase. The V-ATPase is sensitive to nitrate and inhibited by nanomolar concentrations of bafilomycin (Ratajczak, 2000). Therefore, antiport activity can be expected to be absent, or at least significantly reduced, in the presence of these substances. In heavy metal-sensitive *Silene*, Zn uptake activity across the tonoplast was reduced by 42% in the presence of bafilomycin (Chardonnens *et al*., 1999), which clearly indicates a role for the V-ATPase in Zn transport. The severe reduction in uptake due to the addition of protonophores further demonstrates the importance of the proton gradient in Zn transport in these plants. Although many experiments by other authors demonstrate the effect of metals on the proton gradient (Salt and Wagner, 1993; Gries and Wagner, 1998; Gonzales *et al*., 1999), the effect of its absence on metal transport was sometimes omitted, and the possibility remains that transport was at least partly due to another mechanism such as ABC-type transporters which also may be dependent on the Mg ATP present in the assay. This does not hold true for Cd/H^+ antiport activity in oat roots, which was shown to be nitrate sensitive (Salt and Wagner, 1993). However, this transporter had an apparent K_m as high as 5.5 μM, and its physiological relevance remains to be demonstrated (see above).

The data presented in Fig. 2 support the hypothesis that the V-ATPase is of particular importance under stress. Barley root growth was only slightly affected by the addition of either 5 μM $CdCl_2$ or 5 nM bafilomycin A1. However, a combination of both effectors was strongly inhibitory. A plausible interpretation of this phenomenon is that cation antiporter activity, driven by V-ATPase-dependent proton motive force, significantly contributes to the detoxification of Cd by vacuolar compartmentation in barley roots.

The complexity of possible interactions between different transport systems, the variety of substrates for different transporters, and the possible influence of these substrate on the function of the transport system, make

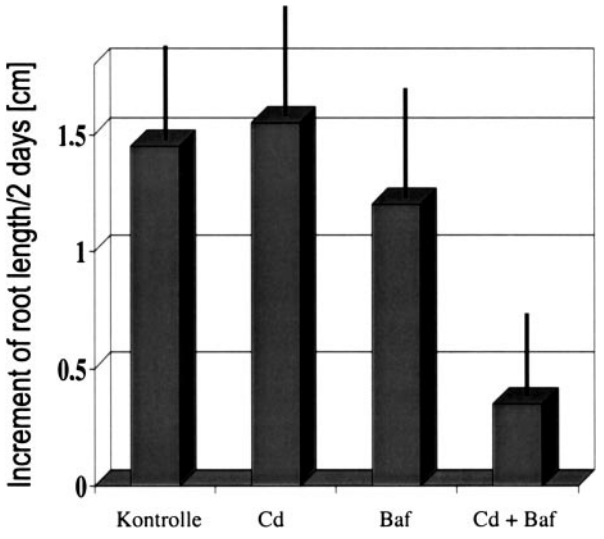

Fig. 2. Increased toxicity of bafilomycin on root growth in the presence of Cd. Root growth of 5-d-old barley seedlings was measured during a 48 h period either in normal hydroponic medium, or medium supplemented with 5 μM $CdCl_2$ or 5 nM bafilomycin A1, or with a combination of both effectors. The growth conditions were essentially as described previously (Brune *et al.*, 1995). The data are means ± SD of 45 roots.

heavy metal membrane transport both a very interesting and a very difficult area of study. Given the fact that many metals may be transported via V-ATPase dependent antiporters, whether as 'natural' or alternative substrates, it is certainly worthwhile to look further into the effect of heavy metals on the structure and function of the V-ATPase, and vice versa.

Cold stress

Many tropical and subtropical plant species are damaged by low, above-freezing temperatures ranging from +10 to −1 °C. In addition to these chilling-sensitive species, most plant species, including those from temperate climates, are generally frost-sensitive or develop frost tolerance only upon hardening. Plant sensitivity towards low temperature, and development of chilling and frost hardiness implicate three vacuolar events related to V-ATPase activity (Yoshida *et al.*, 1999). (1) One of the primary events of chilling injury appears to be an inhibition of V-ATPase activity. (2) As a consequence, the formation of pH gradients is inhibited and probably compartmentation of solutes disturbed. (3) The fluidity of membranes has to be adjusted to low temperatures by an increase of the membrane content of unsaturated fatty acids.

It was an early observation that V-ATPase of chromaffin granules dissociates in the V1 and V0-complexes upon incubation on ice (Moriyama and Nelson, 1989). The occurrence of structural disintegration was confirmed in plants, and shown to account for chilling-induced inactivation of V-ATPase in chilling-sensitive mung bean hypocotyls (Matsuura-Endo *et al.*, 1992). The release of the peripheral subunits was followed by partial degradation of the ATPase complex. Inhibition of V-ATPase was accompanied by cytoplasmic acidosis. In cultured cells of mung beans, a clear correlation was established between chilling sensitivity and cytoplasmic acidosis (Yoshida, 1994). Furthermore, this correlation between stress damage and acidification of the cytoplasm was confirmed in studies comparing V-ATPases from chilling-sensitive species such as mung bean and chilling-tolerant species such as pea (Hotsubo *et al.*, 1998). From the results of that study Yoshida *et al.* concluded that there had to be two types of V-ATPases, i.e. 'mung-bean-type' and 'pea-type', the latter being a cold-tolerant enzyme which shows no V1-dissociation during treatment with chaotropic salts (Yoshida *et al.*, 1999). In contrast, the V-ATPase of the mung bean-type dissociates easily and is a sensitive target of cold stress. At least to some extent, acidification of the cytosol as a consequence of decreased V-ATPase activity accounts for chilling sensitivity, since pH homeostasis within narrow pH ranges is a central element of functional cell metabolism, and directly depends on V-ATPase activity. The situation is complicated because of the presence of the second H^+-pump, the vacuolar pyrophosphatase. It appears that in some species increased H^+ pyrophosphatase activity substitutes for the V-ATPase (Maeshima, 2000).

The role of the PMF for maintaining proper compartmentation may also be significant for chilling- or freezing-induced damage and cold acclimation. The PMF created by tonoplast proton pumps activates secondary active transport through H^+-antiporters. Trans-tonoplast transport of solutes such as sucrose, raffinose and stachyose is mediated by H^+-transport at least in some species and tissues (Greutert and Keller, 1993; Dietz and Keller, 1996). Sugars of the raffinose family are suggested to protect membranes and proteins from chemical damage and to promote intracellular vitrification, thus preventing ice formation and cell death. A low temperature-induced decline of V-ATPase activity and the concomitant decrease in proton motive force may affect solute compartmentation and possibly the hardiness of plants to low temperatures.

The third important aspect of hardening is the modification of lipid composition of the cell membranes which depends on vesicle transport. The importance of the V-ATPase in this process remains to be determined. The V-ATPase inhibitors bafilomycin A-1 and concanamycin inhibited infection of animal cells with the enveloped Semliki Forest virus and the alphavirus Sindbis virus. This study indicates a role of acidic endosomal pH in inducing the fusion activity of the virus particles with the cell membranes (Glomb-Reinmund and Kielian, 1998). It is not known whether luminal acidification is important

for vesicle fusion in plants. If so, the cold-induced inhibition of V-ATPase may also prevent adjustment of lipid fluidity to the low temperature growth condition. In this context it may prove to be interesting that Cd-induced damage of proximal tubules of mammalian kidney is related to a loss of V-ATPase protein with concomitant dissipation of membrane potential and inhibition of endocytosis (Herak-Kramberger *et al*., 1998). Thus, there is some evidence for the importance of V-ATPase in vesicular trafficking from non-plant systems.

Anoxia, acid stress and other adverse growth conditions

Acid stress is the decrease of the cytoplasmic pH either as a consequence of endogenous metabolic activities or of exogenous factors, and can be induced by environmental conditions or artificial treatments, such as anoxia, exposure to acidic air pollutants such as SO_2 or high CO_2 concentrations. The latter experimental approach uses the spontaneous or catalysed hydration of CO_2 with the concomitant release of protons and has been used with both higher plants and algae. These experiments have proven the significance of vacuolar proton pumps in cytoplasmic pH regulation. In the high CO_2-tolerant green algae *Chlorococcum littorale*, V-ATPase amount and activity increased by approximately a factor of two after transfer of the culture to 40% CO_2 (Sasaki *et al*., 1998). Concomitantly, the volume of the vacuoles increased. This response was not seen with *Stichococcus bacillaris* cells, which are sensitive to high CO_2. In leaves, a detailed study of cellular pH regulation also demonstrated the significance of the vacuole for H^+ export. During a 2 min exposure to 2% CO_2, cytoplasmic pH transients in leaf cells were measured *in vivo* by fluorescence emission of pH-indicating dyes. The observed kinetics suggested transport rates of about 1.2 μmol H^+ m^{-2} leaf area s^{-1} from the cytosol to the vacuole (Savchenko *et al*., 2000). Upon withdrawal of the CO_2 from the gas stream, the proton transport was reversed. The H^+ export from the vacuole proceeded at even higher rates. One clue derived from this study is that the vacuolar proton pump can be activated (after addition of CO_2) and deactivated (after removal of CO_2) very quickly, i.e. within a few seconds to minutes. The molecular basis of the fast pH-sensing, signal transduction and regulation of V-ATPase is not understood.

Regulation of H^+-ATPase

As outlined above, tight and precise regulation of V-ATPase is critical for plant development and adaptation to stress. A number of mechanisms have been recognized which alter V-ATPase activity both on a long- and on a short-term basis. However, conclusive and comprehensive concept of V-ATPase regulation is still not available.

Regulation of expression

For the expression of subunits of the vacuolar type H^+-ATPase, developmental control and regulation in response to environmental stresses have been reported, only a few examples of which can be given here. In barley the highest amounts of subunit E mRNA were found in the leaf base, which is the youngest part of the barley leaf, and weaker signals were found in the oldest leaf tissue at the tip. Protein levels were similar in all leaf sections, independent of age (Dietz *et al*., 1995). In cotton (*Gossypium hirsutum* L.) highest mRNA amounts of subunit A of the vacuolar ATPase were found in fibre cells which were rapidly expanding (Smart *et al*., 1998).

Stimulation of the V-ATPase gene expression is a well-known response to environmental stresses in salinity-stressed salt-tolerant plants. Interestingly, mRNA levels of subunit c and subunit E were not modified in *M. crystallinum* plants that were osmotically stressed with mannitol, indicating that the V-ATPase expression is specifically regulated by the ionic, but not by the osmotic component of the salt treatment (Tsiantis *et al*., 1996). In *M. crystallinum*, transcription of subunit E was not modified by exposure of the plants to high light intensities, it was stopped under heat stress conditions, and stimulated by treatment with low temperatures (Fig. 3).

Regulation of the expression of V-ATPase subunits by phytohormones has received some attention. As mentioned above, abscisic acid affected expression of atpv genes in some species. Cytokinin had no effect on V-ATPase activity (Kasai *et al*., 1993). In a gibberellin-deficient tomato mutant, transcript amounts of subunit c and proteins of the subunits A and B increased in response to exogenous gibberelin application (Cooley *et al*., 1999). Thus, atpv gene expression may be under the control of gibberelins, but little is known about the significance of that observation for plant growth under normal and stress conditions. Another open field for future investigations is the signal transduction that regulates the expression of the atpv genes. In *M. crystallinum*,

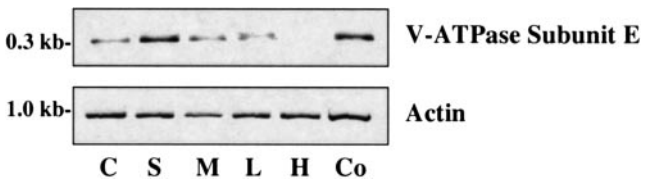

Fig. 3. Transcript amounts of V-ATPase subunit E in leaf tissue of 5-week-old *Mesembryanthemum crystallinum* by RT-PCR. C, Control; S, 6 h salt stress of 400 mM NaCl; M, 6 h osmotic stress with 700 mM mannitol; L, 6 h light stress with doubling of the light irradiance; H, 6 h heat stress at 42 °C; Co, 6 h cold treatment at 4 °C.

induction of atpv E by salinity stress is prevented by feeding the effector of trimeric G-proteins mastoparan to detached leaves (Golldack and Dietz, 2000). These data suggest that G-proteins might be involved in signal transduction pathways affecting the V-ATPase gene expression.

Modulation of ATPase activity

In addition to long-term regulation on the level of expression, V-ATPase activity is subjected to biochemical regulation which may be either fast or slow. Fast modulation of V-ATPase activity is necessary to cope with sudden changes in H^+-dependent secondary transport or cytosolic pH. Application of high CO_2 concentrations to leaves is an effective method to evoke acid stress to leaf cells (Bligny *et al*., 1997). An analysis of leaf tissue with ^{31}P-nuclear magnetic resonance indicated rapid and efficient compensatory H^+-fluxes from the stroma to the cytosol and into the vacuole in the presence of high concentrations of CO_2, or following a period of anaerobiosis. Following sequestration of cytoplasmic phosphate by mannose, which was fed to the leaves, the restoration of normal pH values was prevented after returning to normoxia conditions. Apparently, mannose decreased cytoplasmic ATP levels, making ATP less available for ion pumping across the tonoplast. Some of the parameters known to affect V-ATPase activity are discussed below.

pH and ions: V-ATPase activity is stimulated by Cl^- and reveals maximum activity at neutral pH. With partially purified V-ATPase from oat roots half maximum stimulation was observed at 1 mM Cl^-. ATPase activity declined at elevated pH and was about half at pH 8 as compared to pH 7 treatment (Randall and Sze, 1986). Whereas the Cl^- effect is unlikely to play a role in the regulation of ATPase activity in the cell, the pH optimum is in agreement with the proposed role of V-ATPase in cytosolic pH homeostasis. However, this effect cannot account for the specific adjustment of the cytosol to a pH of close to 7.2. Although both cytoplasmic Cl^-- and H^+-concentrations are affected by stressors such as salinity and anoxia, there is no evidence for a direct regulatory role of anion and proton concentrations.

Adenylates and phosphate: ATP is the substrate of V-ATPase with a K_m of 250–800 μM (Randall and Sze, 1986; Ratajczak, 2000). Accordingly, ATPase activity is stimulated with increasing ATP in the concentration range of 0.5–2 mM. Such fluctuations naturally occur in the cytosol independent of growth conditions. Furthermore, both products of ATP hydrolysis, ADP and inorganic phosphate, are inhibitors of ATP hydrolysis, thereby accentuating the ATP effect: when the cytosolic ATP concentration is high, the cytosolic ADP and Pi-concentrations usually are low, and vice versa. This type of regulation of V-ATPase may explain the transient acidification of the vacuole observed following transfer of leaves from dark to light (Dietz *et al*., 1998). The direct effect of the energy charge on V-ATPase activity may have implications for stress conditions such as drought and anoxia, which are known to inhibit ATP synthesis or ATP consumption. For example, the wheat leaf ATP/ADP ratio dropped from 2.8 to 0.3 when the water potential decreased from −0.5 to −1.8 MPa (Lawlor and Khanna-Chopra, 1984). Although results from total leaf adenylate levels do not allow a deduction for drought-induced ATP/ADP-changes in the cytoplasm, it is likely that such strong changes in total levels are reflected in the cytoplasmic adenylate pool and, thereby, may decrease the activity of the V-ATPase and disturb compartmentation under drought.

Reversible disulphide formation: The V-ATPase of coated vesicles can be inactivated by the formation of an intramolecular disulphide bridge. Atpv A contains two cystein residues, C254 and C532, which have been shown to represent the regulatory disulphide bridge (Feng and Forgac, 1994). Inactivation of plant V-ATPase has been previously shown to occur by SH-group-modifying agents, oxidized glutathione, and during isolation in the absence of dithiothreitol (Hager and Biber, 1984; Dietz *et al*., 1998). *In vitro*, ATP hydrolysis and V-ATPase-dependent acidification of tonoplast vesicles are inhibited upon addition of H_2O_2. Both functions of the oxidized V-ATPase are restored by adding reduced glutathione. Inactivation and reactivation of V-ATPase are accompanied by reversible mobility changes of atpv A and atpv E in non-reducing SDS-PAGE (N Tavakoli and KJ Dietz, unpublished results). Apparently, redox-regulation has the potential rapidly to adjust V-ATPase activity to a given metabolic situation. In this context, it may be particularly interesting that oxidative inactivation of V-ATPase is strongly pH-dependent. Figure 4 shows H^+-pumping by V-ATPase into isolated tonoplast vesicles as monitored by quinacrin-quenching. 2 mM H_2O_2 had no effect on H^+-pumping at pH 6.5, but was strongly inhibitory at pH 8. It may be hypothesized that oxidative down-regulation of V-ATPase is only possible when the cytoplasm is not acidified. The physiological necessity for this phenomenon may be the dominant function of V-ATPase in cytoplasmic pH homeostasis.

Effects of lipids: Delipidated purified V-ATPase has little ATP hydrolysing activity. Upon the addition of asolectin, phosphatidylcholine or soybean phospholipid extract to such preparations, ATPase activity increased close to 50-fold; other phospholipids, fatty acids, mono-, di- or triglycerides added individually stimulated ATPase

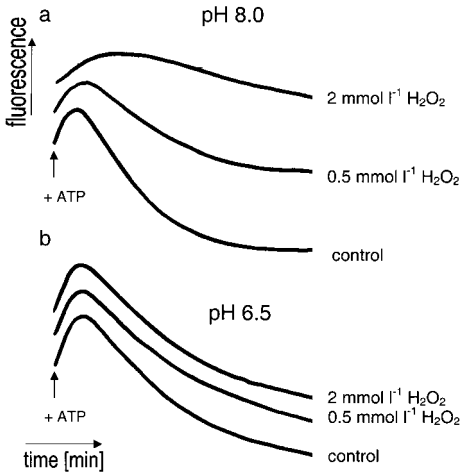

Fig. 4. Oxidative inactivation of V-ATPase-dependent acidification at a pH of the medium of 6.5 or 8.0. Tonoplast vesicles were suspended in a medium containing H_2O_2 at concentrations as indicated and 0.2 mM quinacrine, either at pH 6.5 or 8.0. Proton pumping was activated by adding ATP to the Mg-containing medium. Deflection of the curve to the bottom indicates acidification of the vesicles. 2 mM H_2O_2 inhibited V-ATPase-dependent vesicle acidification at pH 8 but not at pH 6.5 (KJ Dietz and T Mimura, unpublished results).

activity to a varying extent (Yamanishi and Kasamo, 1993). In contrast, glycolipids inhibited phospholipid-activated ATPase activity (Kasamo *et al.*, 2000). Thus, changes in endomembrane lipid composition can have implications on V-ATPase activity. From a comparative determination of lipid composition, electron spin resonance following the introduction of spin-labelled derivatives of stearic acid and V-ATPase activity it was concluded that chilling-induced inhibition of proton pumping across the tonoplast is due to a decrease in the fluidity of the tonoplast caused by glycolipids (Kasamo *et al.*, 2000). Additionally, glycolipids are suggested to induce conformational changes in the V-ATPase complex resulting in decreased proton transport activity.

Modification of subunit composition and modulation by binding proteins: Figure 1 depicts a hypothetical, quite static scheme of the subunit arrangement of the V-ATPase. However, several investigations have shown that the V-ATPase may undergo structural changes in plants. Experiments with the inducible CAM plant *Mesembryanthemum crystallinum* provided evidence that the V-ATPase exhibits a changed subunit composition under salinity (for review see Ratajczak, 2000). The variable size of the membrane particles as an indicator of changing atpv c-number per V-ATPase complex has been discussed above. Altered dimensions of the V1-structure were correlated with the appearance of additional subunits denoted E_i and D_i. By protein sequencing, the latter was shown to be a breakdown product of atpv B and probably occurs in the V-ATPase *in vivo* as well (An *et al.*, 1996). The identification of cDNAs encoding atpv H and a in plants raises questions regarding their function in V-ATPase assembly, structure or regulation. The V-ATPase of bovine clathrin coated vesicles was activated by addition of recombinant atpv H (Zhou *et al.*, 1999). Similar experiments will need to be performed with the plant V-ATPase.

Perspectives

This selective survey of the literature on the topic of the V-ATPase and adaptation of plants to unfavourable growth conditions has yielded sufficient evidence to conclude a significant relevance of functional V-ATPase under stress. However, it also reveals the limits of present knowledge particularly about the structure and regulation of the V-ATPase under normal and stress situations. In the authors opinion, future research should focus mainly on three topics: (1) Elucidation of the functional structure and structural adaptation of the plant V-ATPase: the core structure and basic catalytic mechanism of V-ATPase appear to be conserved from animals to fungi and plants. The availability of gene information for all V-ATPase subunits now allows for the probing of various subunit proteins in order to answer the questions regarding their function in the holocomplex and on developmental, subcellular and stress-specific alterations in complex subunit composition. (2) Co-ordination of expression and function of subunit isoforms: increasing evidence has accumulated that some subunits are present in the plant genome as multiple isogenes (cf. Table 1). For example, subunit isoforms have been described for atpv c, atpv A, atpv E and D (Kawamura *et al.*, 2000). Expression of specific isoforms may be related to plant development and adaptation. Later, the signalling pathways involved in co-ordinated versus non-co-ordinated changes of expression of all subunits in general and of isogenes in particular need to be investigated. (3) Rapid adjustment of V-ATPase *activity*: it appears unlikely that the small set of biochemical mechanisms identified as affecting the V-ATPase activity allows one to understand the rapid regulation of the V-ATPase in all circumstances. Redox-dependent conformational changes may represent one mechanism for fast and efficient regulation of V-ATPase activity. Binding of regulatory subunits or factors, covalent modifications other than disulphide bridge formation and reversible V1/V0-disassembly are examples of possible regulatory mechanisms which should be investigated in more detail.

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