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Review

Structure, function and regulation of the plant vacuolar H^+-translocating ATPase

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Abstract

The plant V-ATPase is a primary-active proton pump present at various components of the endomembrane system. It is assembled by different protein subunits which are located in two major domains, the membrane-integral V_o-domain and the membrane peripheral V_1-domain. At the plant vacuole the V-ATPase is responsible for energization of transport of ions and metabolites, and thus the V-ATPase is important as a 'house-keeping' and as a stress response enzyme. It has been shown that transcript and protein amount of the V-ATPase are regulated depending on metabolic conditions indicating that the expression of V-ATPase subunit is highly regulated. Moreover, there is increasing evidence that modulation of the holoenzyme structure might influence V-ATPase activity.

Keywords: ATP hydrolysis; Gene expression; Holoenzyme structure; Proton transport; Subunit composition; V-ATPase

1. Introduction

The vacuolar H^+-transporting adenosine triphosphatase (V-ATPase; EC 3.6.1.34) is a membrane-bound, primary-active transport protein located at the tonoplast and various other components of the endomembrane system of the plant cell (for reviews see [1–4]). At the tonoplast the V-ATPase is highly abundant, making up 6.5–35% of the total tonoplast protein in different plant species [5,6]. From electron microscopical studies there is evidence for a density of 970–3380 V-ATPase holoenzyme molecules per μm^2 tonoplast area (see [4] and references therein). The V-ATPase is using the energy released during cleavage of the γ-phosphate group of cytosolic ATP to pump protons into the vacuolar lumen, thereby creating an electrochemical H^+-gradient which is the driving force for a variety of transport events of ions and metabolites. Thus, the V-ATPase is extraordinarily important as a house-keeping enzyme to maintain cytosolic ion homeostasis and cellular metabolism. On the other hand, under conditions of environmental stress the V-ATPase functions as a stress response enzyme undergoing moderate changes in expression of subunits and modulations of enzyme structure. Consequently, the V-ATPase is neither a true constitutively expressed housekeeping enzyme nor a true stress enzyme showing strong changes in expression during stress response. Since it is involved in ecophysiological adaptations at the molecular level, the V-ATPase was denominated an 'eco-enzyme' [7].

Investigations performed during the past few years

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clearly showed that depending on the developmental status of the plant and on environmental factors the plant has to cope with, there are changes in V-ATPase amount, activity and subunit composition (for review see [4]). These findings opened new perspectives with respect to the view of regulation of the activity of this important transport protein which are closely related to structural modifications of the holoenzyme. The aim of this review is to provide an overview about the structure and properties of the V-ATPase, its modulation under specific environmental and metabolic conditions, and possible mechanisms of regulation of V-ATPase expression and activity. As a case study for effects of environmental changes on the V-ATPase, the effects of salinity will be discussed in detail.

2. Overall structure of the plant V-ATPase

2.1. Holoenzyme structure

The V-ATPase consists of several polypeptide subunits which are located in two major domains, a membrane peripheral domain (V_1) and a membrane integral domain (V_o). Electron microscopical studies using the negative staining technique revealed a 'head and stalk' structure (see Fig. 1) for the membrane peripheral V_1-domain of the V-ATPase from *Mesembryanthemum crystallinum* [8], *Daucus carota* [9], *Glycine max* [10] and *Beta vulgaris* [11]. This is very similar to the structure of the peripheral F_1-domain of the chloroplastic and mitochondrial F-type ATP synthase (F-ATPase). In analogy to the oligomycin-binding F_o-domain of the F-ATPase the membrane integral domain of the V-ATPase was denominated V_o. V_1 can be dissociated from V_o by incubation of the enzyme in solutions containing chaotropic agents like potassium iodide or at low ionic strength, as shown for the V-ATPase of *Avena sativa* [12,13] and *Be. vulgaris* [14]. For the *Av. sativa* V-ATPase it was demonstrated that the V_1 protein subunits, with the exception of a 42 kDa polypeptide, dissociate as an entire complex from V_o. Lüttge et al. [15] were able to visualize these donut-shaped dissociated V_1-domains of the *Me. crystallinum* V-ATPase by negative contrast electron microscopy.

While the early negative staining studies on the V-ATPase revealed evidence for only a single central stalk connecting the head region and V_o, recently a second peripheral stalk has been discovered by investigation of the *Clostridium fervidus* Na^+-translocating V-ATPase [16]. A peripheral stalk was also detected by re-investigation of the F-ATPase structure using high-resolution electron microscopy and sophisticated methods of image analysis [17]. One subunit located asymmetrically on the top of V_1 of the H^+-translocating V-ATPase visible on electron micrographs is likely to be part of the peripheral stalk [18]. The peripheral stalk is an important component of the rotor-stator model which was presented by Junge et al. [19] to explain the coupling of ATP-hydrolysis and H^+-transport activities of F- and V-ATPases (see Section 3).

The structure of the V_o-domain can be studied by freeze-fracture electron microscopy of tonoplast

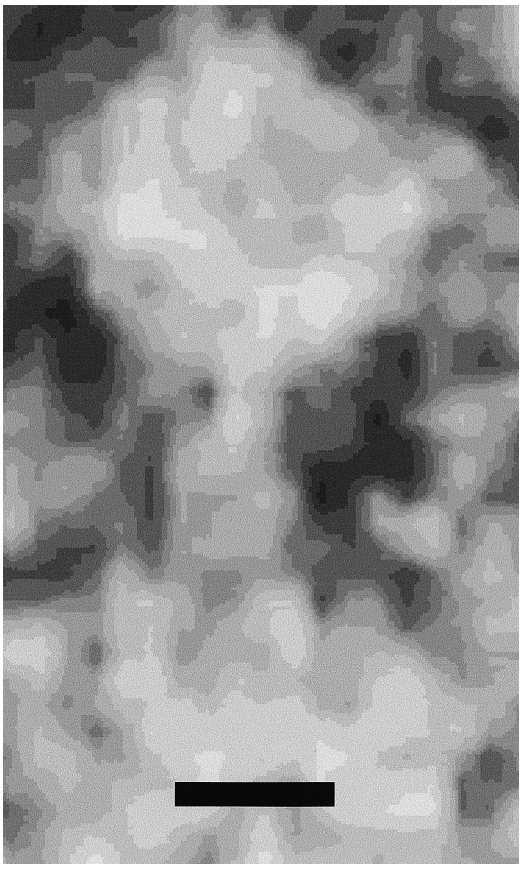

Fig. 1. Electron micrograph of a plant V-ATPase side-view visualized by negative staining of *Mesembryanthemum crystallinum* tonoplast-enriched membrane vesicles (provided by Ilka Emig, Darmstadt, Germany). Bar = 5 nm.

vesicles. From estimates of V-ATPase abundance at the tonoplast arose indirect evidence that most of the intra-membrane particles (IMPs) visible on freeze-fracture replicas of tonoplast vesicles represent V_o-domains of the V-ATPase [5,20]. The identity of a special size class of IMPs as the membrane integral part of the V-ATPase could be shown directly by freeze-fracture analysis of proteoliposomes containing purified enzyme fractions [21]. On freeze-fracture replicas of proteoliposomes containing purified *Kalanchoë daigremontiana* V-ATPase IMPs exhibiting a diameter of 9.1 nm were detected [21]. In contrast, the diameter of IMPs present in proteoliposomes containing reconstituted, partially purified vacuolar H^+-translocating inorganic pyrophosphatase (V-PPase) was around 7 nm. These are the two major size classes of IMPs present in native tonoplast vesicles and in proteoliposomes containing reconstituted total tonoplast protein, indicating that the membrane integral parts of both vacuolar proton pumps can be visualized by freeze-fracturing and that the two enzymes can be distinguished by this method.

2.2. Subunit composition

Early studies on the subunit composition of the V-ATPase [2–4] revealed the existence of three major components exhibiting apparent molecular masses of about 70 kDa (subunit A), 60 kDa (subunit B) and 16 kDa (subunit c). These polypeptides have been found to be subunits of all plant V-ATPases examined (for review see [4]). Subsequent studies revealed that the plant V-ATPase holoenzyme consists of many more protein subunits: up to 10 different subunits were reported for the V-ATPase of *Av. sativa* [13], *Hordeum vulgare* [22] and *Pyrus communis* [23]. This is very close to the number of subunits found to be part of the mammalian and yeast V-ATPase (see Table 1 and [24]). Future investigations will be necessary to determine whether there are additional plant V-ATPase subunits which have not yet been identified. Although the purified and reconstituted *Av. sativa* V-ATPase containing subunits of 70, 60, 44, 42, 36, 32, 29, 16, 13 and 12 kDa was fully competent to hydrolyze ATP and to pump protons into the proteoliposome lumen [25], it cannot be excluded that some additional subunits might have been lost during enzyme purification or were not visible on silver-stained gels.

Sequence information is available for seven of the plant V-ATPase subunits, i.e., subunit A, B, C, D, E, G and c. According to sequence similarity these subunits can be assigned to gene products of the yeast *VMA* (*V*acuolar *M*embrane *A*TPase) genes (for review see [26]). Yeast has turned out to be an excellent model system to identify essential V-ATPase subunits for two reasons. (i) With the exception of *VPH1* [27] and *STV1* [28] encoding homologous proteins, all other V-ATPase subunit genes are present in the yeast genome in a single copy [24]. This is in contrast to the plant genome where genes encoding different isoforms of V-ATPase subunits A, B, G and c have been reported [4,29]. (ii) After disruption of genes encoding essential V-ATPase subunits yeast cells are able to grow in solutions with pH values within a narrow pH range around pH 5.5, but not in medium with pH values higher than 6.5 [30]. This provides a powerful screening system. Table 1 summarizes the yeast and mammalian V-ATPase subunits identified so far, their putative function and the assignment of plant V-ATPase subunits to yeast V-ATPase genes by sequence comparison or according to a similar apparent molecular mass. Subunits A, B, G (b) and c show sequence homology to the well studied F-ATPase subunits β, α, b and c, respectively, and thus, by analogy these subunits are suggested to have similar functions as the respective F-ATPase subunits. This sequence homology of several F-ATPase and V-ATPase subunits and the similarity in holoenzyme structure (see above) led to the assumption that both enzymes evolved from a common ancestor protein [31]. Some special features of V-ATPase subunits found in plants are listed below. Assignment of subunits to different domains of the V-ATPase was performed according to results of V_o–V_1 dissociation experiments [12–15]. A structural model of the plant V-ATPase is given in Fig. 2. Table 1 contains information about plant V-ATPase subunits and references about the identification of V-ATPase subunits in different plant species.

2.2.1. Subunits of the V_1-head

Subunit A. The plant V-ATPase subunit A shows sequence similarity to the catalytic F-ATPase subunit β. The yeast Vma1p protein has been demonstrated

Table 1
Gene products suggested to be part of the yeast and mammalian V-ATPase holoenzyme and assignment of plant V-ATPase subunits to the respective gene products

Yeast gene product	kDa	Sub-unit	Location/putative function	Plant V-ATPase subunits	
				Assignment by partial or complete sequence information	Assignment by apparent molecular mass
Vma1p	68	A	V_1-head/ATP-binding catalytic	*Arabidopsis thaliana* [163]; *Avena sativa* [164]; *Beta vulgaris* (Kirsch, SP Q39442) *Brassica napus* [165]; *Chenopodium* sp. [164]; *Clematis* sp. [164]; *Daucus carota* [166]; *Gossypium hirsutum* [167]; *Hordeum vulgare* (DuPont and Chan, SP Q40002) *Hydrastis* sp. [164]; *Lycopersicon esculentum* [164]; *Mesembryanthemum crystallinum* [91]; *Nicotiana tabacum* [164]; *Phaseolus aureus* [168]; *Zea mays* [95]	63–72 kDa (all plant V-ATPases studied; see [4])
Vma2p	57	B	V_1-head/ATP-binding non-catalytic	*Arabidopsis thaliana* [169]; *Gossypium hirsutum* [170]; *Hordeum vulgare* [92]; *Nicotiana tabacum* [155]; *Mesembryanthemum crystallinum* [91]	52–60 kDa (all plant V-ATPases studied; see [4])
Vma3p	16	c	V_o/H^+-transport	*Arabidopsis thaliana* [93]; Avena sativa [41]; *Beta vulgaris* (Kirsch, SP Q39437) *Clusia minor* [91]; *Daucus carota* [91]; *Gossypium hirsutum* [94]; *Kalanchoë daigremontiana* [171]; *Lycopersicon esculentum* (Cooley et al., SP O24011) *Mesembryanthemum crystallinum* [91,96]; *Nicotiana tabacum* (Kirsch, SP Q40585) *Oryza sativa* [172]; *Phaseolus aureus* (Hung and Pan, SP O22552) *Vigna radiata* [173]; *Zea mays* [95]	16–20 kDa (all plant V-ATPases studied; see [4])
Vma4p	27	E	V_1-stalk/unknown	*Arabidopsis thaliana* [174]; *Gossypium hirsutum* [175]; *Hordeum vulgare* [176]; *Mesembryanthemum crystallinum* [106]; *Spinacia oleracea* [174]	27–32 kDa (*Acer pseudoplatanus* [50]; *Avena sativa* [13]; *Beta vulgaris* [177]; *Hordeum vulgare* [22], *Kalanchoë daigremontiana* [46]; *Pyrus communis* [23])
Vma5p	42	C	V_1-stalk/unknown		37–52 kDa (all plant V-ATPases studied; see [4])
Vma6p	40	M39	V_o/unknown	*Hordeum vulgare* [188]; *Arabidopsis thaliana* [189]	32–36 kDa (*Avena sativa* [13]; *Beta vulgaris* [177]; *Pyrus communis* [23])
Vma7p	14	F	V_1-stalk/unknown		13 kDa (*Avena sativa* [13]; *Hordeum vulgare* [22]; *Pyrus communis* [23])
Vma8p	28	D	V_1-stalk/coupling of ATP-hydrolysis and H^+-transport?	*Arabidopsis thaliana* [38]	30–42 kDa (*Acer pseudoplatanus* [50]; *Avena sativa* [13]; *Citrus sinensis* [67]; *Hordeum vulgare* [22]; *Kalanchoë daigremontiana* [46]; *Mesembryanthemum crystallinum* [46]; *Pyrus communis* [23])
Vma10p	13	M16 G	V_1-stalk?/coupling of ATP-hydrolysis and H^+-transport?	*Nicotiana tabacum* [29]	13 kDa (*Avena sativa* [13]; *Hordeum vulgare* [22]; *Pyrus communis* [23])

Table 1 (*continued*)

Yeast gene product	kDa	Sub-unit	Location/putative function	Plant V-ATPase subunits	
				Assignment by partial or complete sequence information	Assignment by apparent molecular mass
Vma11p	17	c′	V_o/H^+-transport?		
Vma16p	23	c″	V_o/H^+-transport?		
VPH1,STV1	95	M115	V_o/assembly? targeting?		95–115 kDa (*Acer pseudoplatanus* [50]; *Avena sativa* [44]; *Beta vulgaris* [177]; *Hordeum vulgare* [22])
		M20 (a)	V_o/unknown		
		M9.2	V_o/unknown		

For information about yeast and mammalian V-ATPase subunits see [24] and references therein. The bovine subunit M9.2 was described by Ludwig et al. [162], for subunits c′ and c″ see [26]. SP, SwissProt protein database accession number.

to be homologous to the plant V-ATPase subunit A. It contains a nucleotide-binding site, and the binding of the adenine analogue 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole [32], of fluorescein 5′-isothiocyanate [33] or of *N*-ethylmaleimide [32,34] to subunit A inhibits ATP-hydrolysis activity. Thus, subunit A seems to be the catalytic subunit of the V-ATPase. The molecular mass calculated from amino acid sequences ranges from 68.5 to 68.8 kDa in different plant species.

Subunit B shows sequence similarity to the F-ATPase subunit α. The yeast Vma2p protein has been shown to be a homologue of the plant V-ATPase subunit B. It contains a nucleotide-binding site and binding of the photoactivated ATP-analogon 3-*O*-(4-benzoyl)benzoyladenosine-5′-triphosphate does not inhibit ATP-hydrolysis activity of the V-ATPase in a simple competitive manner [35]. This indicates that subunit B is a substrate-binding non-catalytic subunit. The deduced molecular mass ranges from 53.7 to 54.7 kDa.

For the F-ATPase the presence of three β and three α subunits in F_1 was demonstrated by X-ray crystallography [36]. Crystals of the V-ATPase head which can be used for crystallography are not available yet, but taking in account the homology of subunits A and B with the F-ATPase subunits β and α, the similarity of holoenzyme structure of both enzymes and a subunit A:B ratio of 2.93:3.22 which was determined by biochemical analysis of the bovine V-ATPase subunit stoichiometry [37] it can be suggested that the V_1-head contains three copies of each subunit A and B (but see Section 4.2).

2.2.2. Subunits of the V_1-stalk

Subunit C. Polypeptides exhibiting apparent molecular masses on polyacrylamide gels ranging from 37 to 52 kDa have been suggested to represent subunit C. The function of subunit C is unknown but it might be involved in stabilization of V_1. Recently, the C subunit of *Ho. vulgare* and *Arabidopsis thaliana* has been cloned, showing sequence similarity to the yeast *VMA5* gene.

Subunit D of *Ar. thaliana* (homologue to the Vma8p) was recently cloned [38]. The polypeptide has a molecular mass of 29.1 kDa. Analysis of the yeast subunit D revealed structural similarities to the γ-subunit of the F-ATPase although no significant sequence similarity was detected [39]. Thus, subunit D could be involved in the connection of V_o and the V_1-head and participate in coupling of ATP-hydrolysis and H^+-transport (see Section 3.3).

Subunit E. Cloning of subunit E genes from plants revealed sequence similarity to the yeast Vma4p protein. The function of the polypeptide which has a molecular mass of 26.2–27.1 kDa in different species is unknown.

Subunit F. Sequence information for the plant V-ATPase subunit F (homologous to the yeast Vma7p protein) is not available. A polypeptide exhibiting an apparent molecular mass of 13 kDa found in several plant species might represent this subunit, the function of which is unknown.

Subunit G. A plant V-ATPase subunit homologous to the yeast Vma10p protein was recently cloned from two plant species, and the polypeptide encoded by these clones exhibited a molecular mass of

12.5 kDa. The *Nicotiana tabacum* subunit G gene was shown to restore the wild-type phenotype of a yeast *VMA10* mutant [29]. Subunit G exhibits significant sequence similarity to the F-ATPase b-subunit although it is shorter than F-ATPase b-subunit and does not contain an apparent transmembrane domain at its N-terminus [40]. In analogy to the F-ATPase b-subunit, subunit G is a good candidate to be part of the peripheral V-ATPase stalk which might be involved in coupling of ATP-hydrolysis and H^+-transport. Interestingly, a 12 kDa polypeptide which might represent subunit G of the *Av. sativa* V-ATPase remains at the V_o-domain after dissociation of V_1 [13] and a 12 kDa *Me. crystallinum* polypeptide cross-reacting with an antiserum against *Ni. tabacum* subunit G was found in the holoenzyme but not in a fraction of dissociated V_1-subunits (Emig, I., personal communication). This indicates a tight contact of subunit G with the V_o-domain.

2.2.3. Subunits of the V_o

Subunit c (homologous to the Vma3p protein and to subunit c of the F-ATPase) is the main component of the V_o-domain. Subunit c genes from several plants have been cloned and deduced amino acid sequences revealed polypeptides exhibiting molecular masses ranging from 16.6 to 16.7 kDa. Subunit c is a highly hydrophobic protein containing four membrane-spanning domains [41] and is suggested to be directly involved in H^+-transport. In the yeast subunit c ^{137}Glu, which is located in the membrane-spanning domain IV, has been demonstrated to be essential, since replacement of this amino acid by other amino acids, with the exception of aspartate, abolished V-ATPase activity [42]. This negatively charged amino acid is suggested to be the binding site of *N*,*N*′-dicyclohexylcarbodiimide (DCCD), which inhibits V-ATPase activity. DCCD-binding studies revealed that there are at least six c-subunits per V-ATPase holoenzyme [43]. However, binding of a single DCCD molecule per V-ATPase is sufficient to abolish V-ATPase activity indicating a high degree of cooperativity between the different c-subunits ([43]; see also Section 3.3).

Polypeptides of 32–36 kDa found to be part of the V_o-domain of several plant V-ATPases might be analogues to the yeast Vma6p protein, the function of which is unknown.

Polypeptides of 95–115 kDa have been demonstrated to co-purify with V-ATPase subunits in several, but not in all, plant species. It is unclear whether these polypeptides are analogues to the VPH1/STV1 proteins of the yeast V-ATPase. Both polypeptides do not exhibit sequence similarity to F-ATPase subunits and seem to be unique to the V-ATPase. A possible function of these proteins is an involvement in V-ATPase assembly [27] or targeting of the V-ATPase to the vacuole [28]. The finding that a 100 kDa polypeptide was associated with a fraction of V_o-domains lacking V_1 of the *Av. sativa* V-ATPase, but that this same polypeptide was not detected in the active V-ATPase holoenzyme might suggest a role of the 100 kDa polypeptide in V-ATPase assembly [44].

From the molecular masses of the plant V-ATPase subunits mentioned above and taking into account that three copies of subunits A and B and six copies of subunit c might be present per V-ATPase holoenzyme while all other subunits are present as single copies, a molecular mass of approximately 730 kDa can be calculated for the plant V-ATPase holoenzyme. This is close to the molecular masses determined by size-exclusion chromatography for the V-ATPase holoenzymes of *Z. mays* (400 kDa; [45]), *K. daigremontiana* (510 kDa; [46]) and *Av. sativa* (650 kDa; [13]). However, it has to be pointed out that in preparations from different plant species the subunit composition of the V-ATPase holoenzyme was not identical. The simplest explanation for this could be that the purity of the preparations analyzed varied or that some subunits were lost during enzyme purification. On the other hand, if the subunit composition really varies between species, this could have an impact on holoenzyme properties leading to variations in enzyme activity.

3. Characteristics of V-ATPase activity

3.1. Effectors of V-ATPase activity

Initial evidence for V-ATPase activity came from studies of a membrane-bound H^+-translocating ATPase in the vacuole-like lutoids of *Hevea brasiliensis* [47,48]. The pH optimum of enzyme activity was in the range of pH 7.5 to 8.0 and activity was stimu-

lated by Cl^-. Later it turned out that anion stimulation (Cl^-, $Br^- > HCO_3^-$; [34]) is a common feature of plant V-ATPases. Using different inhibitors, the activity of the V-ATPase can be distinguished from the activity of other ATP-hydrolyzing H^+-pumps present in the plant cell [2]. In contrast to the plasma membrane H^+-ATPase (P-ATPase), which is inhibited by orthovanadate, which prevents the formation of a phosphorylated intermediate state of the functional cycle [49], most of the V-ATPases studied so far are vanadate-insensitive. There are only two examples for vanadate-sensitive V-ATPases: a H^+-pump at the tonoplast of *Acer pseudoplatanus* cells which seems to operate in a P-ATPase-like manner including the formation of a phosphorylated intermediate [50] and the V-ATPase present in the tonoplast of *Citrus limon* fruit [51,52]. Both the F-ATPase and the V-ATPase activities are sensitive to nitrate, however in contrast to the mitochondrial F-ATPase, the V-ATPase activity is insensitive to azide [3]. Thus, nitrate-sensitive, azide-resistant ATP-hydrolysis activity was used to characterize V-ATPase activity. Members of the macrolide antibiotic family have been determined to be more specific inhibitors of the V-ATPase. Bafilomycin A_1 inhibits V-ATPase activity in nanomolar concentrations [53] and Concanamycin A [54] is even more effective (by a factor of ten). While macrolide antibiotics also inhibit the P-ATPase at 1000-fold higher concentrations as needed for inhibition of the V-ATPase, they do not affect F-ATPases [54]. The mechanism of inhibition of the V-ATPase by Bafilomycin A_1 is unclear. From studies of proteoliposomes containing reconstituted V_o-subunits there is some evidence that Bafilomycin A_1 might bind to V_o. In contrast to the F-ATPase F_o-domain the native V-ATPase V_o-domain does not exhibit passive proton conductivity after dissociation of V_1 [55]. For unknown reasons after reassembly of partially purified V_o-subunits in proteoliposomes proton conduction is measurable. Since Bafilomycin A_1 blocks H^+-translocation of these reassembled V_o-domains lacking V_1-subunits, it was suggested that Bafilomycin A_1 might bind to V_o [56,57]. Although Bafilomycin A_1 inhibition of V-ATPase activity is generally very effective, it does not seem to inhibit all V-ATPases in a similar manner. The V-ATPase of the *Ci. limon* fruit tonoplast [51] appeared to be Bafilomycin A_1-insensitive and treatment of *Ho. vulgare* leaves with methyljasmonate decreased Bafilomycin A_1-sensitivity of the V-ATPase [58]. Moreover, there is evidence from investigation of tonoplast-enriched membrane vesicles isolated from *Ni. tabacum* leaves that the degree of Bafilomycin A_1 inhibition of V-ATPase activity decreases with increasing incubation temperature used during the activity assay, while nitrate-sensitivity of the same membrane vesicle preparations was constant over a broad temperature range (E. Fischer-Schliebs, personal communication). This different behavior of the two inhibitors might be explained by different targets of inhibitor binding leading to V-ATPase inactivation. From patch-clamp studies of whole vacuoles there is evidence that the site of nitrate action is at the cytoplasmic side of the V-ATPase since nitrate applied to the lumenal side had no effect on V-ATPase activity [59]. Dschida and Bowman [60] suggested that inhibition of the *Neurospora crassa* V-ATPase activity by nitrate is due to oxidation of critical cysteine residues in V_1. As mentioned above the site of Bafilomycin A_1 action seems to be V_o, and thus it is probable that the properties of the lipid environment at different temperatures might influence the efficiency of Bafilomycin A_1 binding to V_o.

In addition to nitrate and macrolide antibiotics several other chemicals, mainly protein modifying agents (some of which are mentioned in Section 2.2) have been shown to inhibit V-ATPase activity. These inhibitors have been recently reviewed in [4]. Interestingly, anti-calmodulin drugs (W5, W7, Calmidazolium) and the calcium channel antagonists Verapamil and Diltiazem have been shown to inhibit V-ATPase activity directly [61]. Thus, the V-ATPase itself might be involved in the regulation of metabolic responses as a component of Ca^{2+}-dependent signal transduction chains.

3.2. Enzyme kinetics and turnover numbers

A nucleotide specificity of $ATP \gg GTP > NTP$ is common for plant V-ATPases [2,62,63]. ATP-divalent cation complexes ($MgATP^{2-} \geq MnATP^{2-}$, $ZnATP^{2-} \gg CaATP^{2-}$, $CoATP^{2-}$) [63,64] function as substrates. K_m values ranging from 200 to 810 μM have been reported for different plant V-ATPases [34,62,65–69]. For the substrate hydrolysis activity of the *K. daigremontiana* V-ATPase two K_m

values (770 μM and 2 μM) have been found [70], indicating the presence of at least two different catalytic centers at the enzyme or cooperativity between nucleotide binding sites.

By relating nitrate-sensitive V-ATPase activity to immunologically determined V-ATPase protein amount in tonoplast vesicles isolated from *Me. crystallinum* leaves [71] a turnover-number of 50 ATP s^{-1} was calculated [15]. This is close to the value of 30 ATP s^{-1} which can be calculated from the activity of purified *K. daigremontiana* V-ATPase after reconstitution in proteoliposomes [70]. With tonoplast-enriched membrane vesicles isolated from *Av. sativa* [13] and *Vigna radiata* [72] transport rates of 60 and 90 H^+ s^{-1}, respectively, have been determined.

3.3. Coupling of ATP-hydrolysis and H^+-transport

The V-ATPase actually exhibits two distinct activities which can be measured by application of different techniques. ATP-hydrolysis activity is generally calculated by determination of the phosphate released during ATP-hydrolysis [73,74]. H^+-transport activity can be estimated by monitoring fluorescence quenching or absorption changes of the dyes quinacrine or acridine orange due to pH-gradient-driven dye accumulation in vacuoles, tonoplast vesicles or proteoliposomes [2,75,76]. Both activities can be measured simultaneously in one assay by applying the method of Palmgren [77] using enzyme systems that couple ATP-hydrolysis with the oxidation of NADH for the determination of ATP consumption, while H^+-accumulation is monitored by determination of acridine orange absorption changes. The advantage of the simultaneous measurement of both activities in one assay is the avoidance of artifacts which can occur in the case of separate measurements of both activities if different aliquots of a given membrane-vesicle sample exhibit different proportions of inside-out and right-side-out vesicles.

From investigations on vacuoles and tonoplast vesicles isolated from crassulacean acid metabolism (CAM) plants for thermodynamic analyses, a stoichiometry $2H^+_{transported}/ATP_{hydrolyzed}$ was suggested for the coupling of H^+-transport and ATP-hydrolysis activity [78,79]. The same coupling ratio was determined for the *Be. vulgaris* V-ATPase [80]. Interestingly, from patch-clamp studies on *Be. vulgaris* vacuoles there is evidence for a variable H^+/ATP stoichiometry: the coupling ratio decreased from 3.28 to 1.75 when the pH gradient between the cytoplasm (pH 7.6) and the vacuole was increased from 2.8 to 4.7 [81]. A lower coupling ratio, i.e., a lower number of protons pumped per ATP hydrolyzed, would benefit transport of protons into the vacuole against steep H^+-gradients for thermodynamic reasons. The results of Davies et al. [81] suggest that the coupling of the two V-ATPase activities is variable and can be adapted to the actual pH-gradient across the tonoplast to reach vacuolar pH values of 3 or lower.

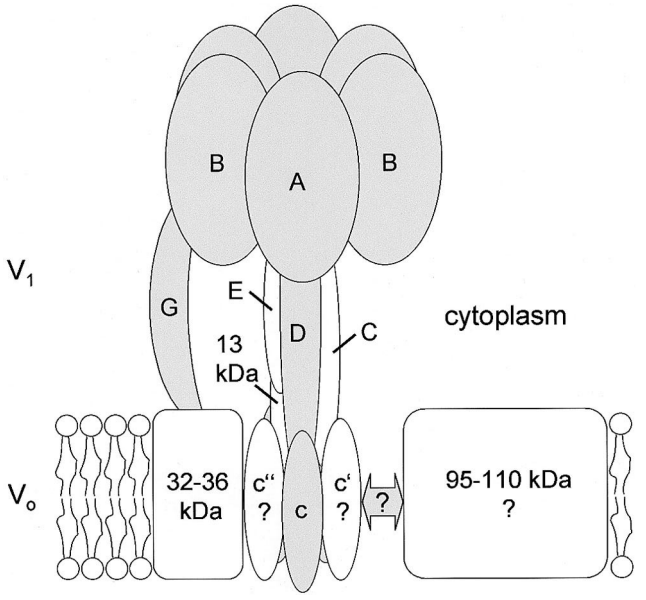

Fig. 2. Structural model of the plant V-ATPase based on analogy of V-ATPase subunits to F-ATPase subunits (subunits given in gray) and information obtained by dissociation of the V_o- and V_1-domain (for details see text and Table 1). For plant V-ATPase subunits which have not yet been sequenced, apparent molecular masses are given. Question marks indicate subunits which have not unequivocally shown to be part of the active V-ATPase holoenzyme.

Recently Junge et al. [19] presented a functional model of the F-ATPase to explain the mode of coupling between ATP-synthesis/hydrolysis and H^+-translocation. The main feature of the model is a rotor-stator mechanism, in which a revolver-like ring of c-subunits (proteolipids) in the F_0-domain and the γ-subunit (which is homologue to the V-ATPase D subunit; see Fig. 2) of the central stalk represent the rotor, while additional F_o subunits

linked to the F-ATPase head by a second peripheral stalk build up the stator. A negatively charged amino acid residue of the F-ATPase subunit c located in the center of the lipid bilayer seems to be essential for H^+-transport. In the yeast V-ATPase subunit c this amino acid might be ^{137}Glu. The rotor performs Brownian movements which after protonation of the negatively charged group is only possible in one direction. This leads to a rotation of the rotor relative to the stator. Rotation of the γ-subunit, exhibiting an asymmetrical structure, in the center of the F_1-head is suggested to lead to conformational changes of β- and α-subunits which are prerequisite for ATP-synthesis or hydrolysis. Since the F-ATPase and the V-ATPase are structurally closely related, a similar coupling mechanism might be present in the V-ATPase. According to the model a change in the number of c-subunits in the proteolipid ring or incorporation of different c-subunit isoforms exhibiting different numbers of H^+-binding sites could act as gear shift mechanism controlling the coupling ratio of the enzyme. Such a control mechanism could be useful to adopt the coupling ratio to the actual requirements for H^+-transport under certain environmental conditions (see Section 4).

3.4. Post-translational regulation of V-ATPase activity

Plant V-ATPase activity has been suggested to be regulated by several different mechanisms, i.e., phosphorylation of V-ATPase subunits [82–86], modification of the redox state of the enzyme by oxidation and reduction of essential sulfhydryl groups present in V-ATPase subunits A and B [60,87,88]. Also changes of the availability of the V-ATPase substrate $MgATP^{2-}$ in the cytoplasm has been proposed to be an important factor for regulation of V-ATPase activity [89]. Since the V-ATPase V_o-domain is embedded in the lipid bilayer, the lipid environment of the enzyme can be expected to be crucial for enzyme activity. Yamanishi and Kasamo [90] demonstrated the inactivation of the purified V-ATPase by removal of lipids using ion-exchange chromatography. The purified, partly delipidated enzyme contained 10–15 sterol molecules and 20–30 glycolipid molecules while phospholipids could not be detected. Enzyme activity could be restored by addition of the phospholipid mixture asolectin and different species of phospholipids. This indicates that the phospholipids present in the liquid crystalline phase of the tonoplast are necessary for V-ATPase activity.

3.5. Possible role of subunit isoforms

As mentioned above (see Section 2.2), small gene families have been detected in the plant genome for most plant V-ATPase subunits. Two isoforms of subunit A have been found in *Av. sativa*, *Chenopodium* sp., *Clematis* sp., *D. carota*, *Go. hirsutum*, *Hydrastis* sp., *L. esculentum*, *Ni. tabacum* [31] and *Me. crystallinum* [91]. Two isoforms of subunit B are present in *Ho. vulgare* [92] and *Me. crystallinum* [91]. Recently, the V_1-stalk subunit G has been demonstrated to exist in two isoforms in *Ni. tabacum* [29]. For the membrane-integral subunit c four different isoforms have been cloned in *Ar. thaliana* [93] and *Av. sativa* [41], while two subunit c isoforms have been identified in *Go. hirsutum* [94], *Me. crystallinum* [91] and *Z. mays* [95]. A common feature of all subunit c isoforms is a high degree of sequence similarity in the coding regions, while they differ significantly in the non-coding regions. Regulation of V-ATPase activity by expression of different subunit isoforms in various tissues or under certain environmental conditions would be an intriguing possibility. First evidence for a tissue-specific and developmentally regulated expression of subunit c isoforms came from studies on *Go. hirsutum* [94]. Subsequently, in *Me. crystallinum* the subunit c *Vmac1* transcript was found to be preferentially expressed in leaves [96]. In *Ar. thaliana* two subunit c genes (*AVA-P1* and *AVA-P2*) are constitutively expressed in all tissues tested, while message levels of *AVA-P3* and *AVA-P4* are very low and *AVA-P3* expression seems to be restricted to the root and shoot apex (X. Lin, H. Sze, personal communication). Taking into account the different functions of the V-ATPase in cellular metabolism it can be hypothesized that some of the isoforms are 'house-keeping' forms while others are expressed under conditions of environmental stress. Moreover, different subunit isoforms might be incorporated into V-ATPases located in different intracellular membranes (see Section 6). However, evidence for information on the expression of different isoforms as a response to environmental stress or

on the presence of different isoforms at different cellular membranes is lacking and further work is needed to confirm this hypothesis.

4. Responses of the V-ATPase to changes in environmental and metabolic conditions

4.1. Changes in message and protein amount and enzyme activity

The plant vacuole has essential functions for the maintenance of cellular metabolism due to its role in long-term storage of toxic ions, in long-term or short-term storage of minerals or organic acids and in cytoplasmic pH and Ca^{2+} homeostasis. Thus, it can be expected that the activity of the V-ATPase, which is the major H^+-pump of the tonoplast, is modulated to cope with environmental and metabolic changes. Despite the importance of the enzyme for vacuolar energization, detailed studies on V-ATPase responses at the levels of transcript, protein and activity are only available for conditions of salt stress (see Table 2) and to a lesser extent to low temperature stress conditions.

The message amount of all V-ATPase subunits analyzed seems to increase in response to salt stress. A salt-induced 2–4-fold increase in subunit A message was first reported to occur in cultured cells of *Ni. tabacum* [97]. An increase in subunit A message was subsequently detected after salt treatment of intact plants of *L. esculentum* [98–100], *Be. vulgaris* [101,102] and *D. carota* [103]. In cultured cells of *D. carota*, message levels for subunits A and c transiently increased 2–3-fold compared to controls 4 days after treatment with 100 mM NaCl [104]. An increase in subunit c transcript was found in *Ar. thaliana* [93], *Be. vulgaris* [101,102], *D. carota* [103] and *Me. crystallinum* [91,96]. In *Me. crystallinum* a few hours of salt stress led to an increase in subunit c message while transcript abundance for subunits A and B remained constant [91]. This was interpreted as evidence for non-coordinated regulation of V-ATPase subunits. When salt treatment was continued for several days, in addition to subunit c mRNA, levels of mRNA for subunits A and B were also increased [105]. Interestingly, the degree of message accumulation was different for the three V-ATPase subunits. After 12 days of salt treatment subunit A, B and c message amount increased by factors of 2, 12 and 5, respectively. The observation that the transcript of the V-ATPase stalk subunit E of *Me. crystallinum* was unchanged after 48 h of salt stress [106] is another suggestion of non-coordinated expression of V-ATPase subunits. However, salt-induced expression of V-ATPase subunits has been found to be coordinated in *Be. vulgaris* [101] and *D. carota* [103].

Information about the effect of salt-stress on the

Table 2
Salinity-induced changes in V-ATPase message and protein amount and activity in various species (→, no change; ↓, decrease; ↑, increase)

Species	Message amount	V-ATPase amount	ATP-hydrolysis activity[a]	H^+-transport activity
Atriplex nummularia				↑ [178]
Mesembryanthemum crystallinum	↑ [91,96,105]	↑ [71]	↑ [46,62,66,112]	↑ [62,66,112]
Sorghum bicolor			↑ [179]	
Hordeum vulgare		→ [108,109,180]	→ [109]	↑ [109,180,181]
Arabidopsis thaliana	↑ [93]			
Beta vulgaris	↑ [101,102]			
Vigna radiata			↑ [182]	
Nicotiana tabacum	↑ [97]	↓ [107]	↑ [107]	↑ [107]
Lycopersicon esculentum	↑ [98–100]		↑ [183]	
Daucus carota	↑ [103,104]	→ [104]	→ [184], ↓ [104]	↑ [104]
Spartina townsendii			→ [179]	
Helianthus annuus			→ [111,185]	↑ [111]
Gossypium hirsutum				→ [186]
Acer pseudoplatanus			→ [187]	

[a]Since in most of the studies mentioned the V-ATPase protein amount was not determined, ATP-hydrolysis activity does not reflect specific enzyme activity but hydrolysis activity related to total tonoplast protein.

amount of the V-ATPase holoenzyme protein is only available for few species. While in *Ni. tabacum* the staining intensity of subunit A on Western blots decreased due to salinity [107] salt treatment of *Ho. vulgare* plants [22,108,109] and cultured cells of *D. carota* [104] had no effect on V-ATPase protein amount. Salt-induced changes of the protein amount in *Me. crystallinum* leaves were monitored using different immunological techniques, i.e., radial immunodiffusion [71], quantitative immunoprecipitation of the V-ATPase holoenzyme and immunoelectron microscopy of tonoplast vesicles [110]. It turned out that V-ATPase in tonoplast-enriched membrane vesicle fractions increased by a factor of 2.5 during 8 days of irrigation with 0.4 M NaCl. When salt was removed from the root medium the V-ATPase protein amount decreased to reach the V-ATPase level in tonoplast vesicles isolated from well-watered control plants within 48 h, indicating a rapid turnover of the V-ATPase holoenzyme [71].

There is a considerable amount of data available for the behavior of ATP-hydrolysis and H^+-transport activity of the V-ATPase in response to salinity (see Table 2). From these data it can be concluded that at least in halophytes and salt-tolerant plants salinity induces an increase in V-ATPase activity. However, in most of the studies the activity of the enzyme was related to total tonoplast protein, and thus does not represent the true specific activity of the enzyme. In *Me. crystallinum*, both the activity and the protein amount of the V-ATPase was determined opening the possibility to calculate specific activities which were almost identical for the V-ATPases isolated from controls and salt-treated plants [71].

An interesting but unexpected finding was that in tonoplast vesicles from cultured cells of *D. carota* salt stress increased H^+-transport activity by 60% while ATP-hydrolysis activity of the V-ATPase decreased by 20% [104]. Similar results were obtained studying tonoplast vesicles from *Helianthus annuus* [111] and *Ho. vulgare* [109] where salinity increased H^+-transport activity while ATP-hydrolysis activity of the V-ATPase remained unchanged. After salt treatment of intact plants (E. Berndt, R. Ratajczak, unpublished) and cultured cells [112] of *Me. crystallinum* H^+-transport activity related to total tonoplast protein increased to a larger degree compared to ATP-hydrolysis activity. These data indicate that salinity conditions might alter the coupling ratio of the V-ATPase (see Section 4.4). The finding that in tonoplast vesicles from *D. carota* the coupling ratio of the V-ATPase changed after the onset of salt treatment, while the coupling ratio of the V-PPase at the same membrane did not, supports the hypothesis that the observed change in V-ATPase coupling ratio is a result of differences in intrinsic changes of the V-ATPase and does not result from changes of H^+-permeability of the tonoplast [104].

Growth of plants at low temperatures seems to have an impact an V-ATPase activity. When chilling-sensitive *V. radiata* seedlings were grown at temperatures below 10°C, H^+-transport activity of the V-ATPase decreased to a very low level [113]. This effect was not observed by cold-temperature stress of chilling-insensitive *Pisum sativum* seedlings. The authors could demonstrate that the effect observed in *V. radiata* was not due to permeability changes of the tonoplast, but to V-ATPase inactivation. Recovery of the V-ATPase activity was not dependent on de novo protein synthesis [114], indicating a mechanism for regulation of the V-ATPase at the protein level. This mechanism could be a reversible dissociation of the V_o- and V_1-domains of the V-ATPase. This suggestion was supported by the finding that after incubation of *V. radiata* hypocotyls for 3 days at 0°C the amount of several V_1-subunits was decreased while the amount of subunit c was not altered [72]. Future work is needed to show whether dissociation and reassociation of the two major domains V_o and V_1 might be an important tool in general to regulate V-ATPase activity under certain metabolic conditions.

4.2. Structural changes of V_1

Structural modifications of the V-ATPase holoenzyme due to variations in environmental and metabolic conditions have been found in different plant species. The largest data set, however, is available from studies on the V-ATPase of the halophyte *Me. crystallinum* during the salt-induced shift from C_3-photosynthesis to CAM. The V-ATPase energizes both the transport of Na^+ and Cl^- into the vacuole and nocturnal malate accumulation in the vacuole, which is a main feature of CAM. Thus, using *Me.*

crystallinum responses of the V-ATPase to different environmental and metabolic conditions can be studied. Negative contrast transmission electron microscopy and image analysis of side views of *Me. crystallinum* V-ATPase (see Fig. 1) indicated that the dimensions of V_1 are different in tonoplast vesicles isolated from plants in the C_3-state of photosynthesis and from plants after induction of CAM by salinity [4]. The V_1-head diameter of the *Me. crystallinum* C_3-ATPase was 9.8 nm which is in good agreement with the head diameters of other C_3-plants (*Be. vulgaris*: 9.0 nm [11]; *D. carota*: 9.4 nm [9]), while the diameter of the *Me. crystallinum* CAM-V-ATPase was 11.8 nm. In addition, the V_1-stalk diameter was 2.0 nm and 3.1 nm in the C_3-V-ATPase and the CAM-V-ATPase, respectively [4]. These structural changes of the V-ATPase holoenzyme are correlated with modification of the subunit composition of the V-ATPase occurring during the shift from C_3-photosynthesis to CAM. In this respect, the appearance of two polypeptides exhibiting molecular masses of 28 (E_i) and 32 kDa (D_i) during salt-induced C_3-CAM shift in tonoplast vesicles isolated from *Me. crystallinum* is of special interest [46]. The appearance of these polypeptides was demonstrated to be correlated to the expression of CAM and not to exposure of plants to high salt. By immunoprecipitation experiments it could be shown that these polypeptides are closely associated with the V-ATPase holoenzyme [71] and after dissociation of the V-ATPase holoenzyme using chaotropic agents they were found in the fraction containing V_1-subunits [15]. The N-terminal amino acid sequences of the polypeptides D_i (XGGEEDNFAIVFA...) and E_i (XPGGCGAGGGCXGGA...) were determined several years ago [115]. At that time no homologous amino acid sequences of other proteins were found in protein data bases. However, after the amino acid sequence of V-ATPase subunit B (isoform 2) of *Ho. vulgare* [92] was published, it turned out that the known 12 amino acids of D_i perfectly fit amino acids 194–205 of *Ho. vulgare* B-subunit. Sequence analysis of a partial *Me. crystallinum* cDNA clone revealed that the *Me. crystallinum* and *Ho. vulgare* B-subunit amino acid sequences are identical in this region [91]. Thus, it was suggested that D_i represents a proteolytically processed B-subunit [116]. By analysis of phenol extracted proteins from *Me. crystallinum* leaves in the CAM-state it could be demonstrated that D_i occurs in vivo and is not produced as an artifact during tonoplast vesicle isolation [116]. Proteolytic processing is restricted to subunit B since degradation products of other subunits have not been detected. This processing of subunit B might have an impact on V-ATPase properties, since the *Me. crystallinum* CAM-V-ATPase (containing D_i) is less sensitive to detergent treatment [68] and incubation at high concentrations of malate [15]. Moreover, proteolytic processing of V_1-head subunits might be the reason for the intriguing finding that the *Me. crystallinum* V-ATPase head structure is not always a hexamer of subunits [117] as it was proposed from determinations of subunit stoichiometry (see Section 2.2). In addition to hexameric structures pentameric heads have been detected. This could be explained by different head subunit stoichiometries, e.g., A_3B_3 for the hexamer and A_2B_3 or A_3B_2 for the pentamer after proteolysis of one V_1-head subunit. Another explanation might be that the pentamers represent a stage of holoenzyme assembly.

The appearance of additional polypeptides of about 30 kDa associated with the V-ATPase is not restricted to *Me. crystallinum*. After salt treatment of *Citrus sinensis* a 35 kDa polypeptide appeared which was assumed to be a proteolytic fragment of subunit A [118] and cultivation of *K. daigremontiana* plants at elevated temperatures led to the formation of a 35 kDa polypeptide in tonoplast-enriched membrane vesicles cross-reacting with V-ATPase antisera [119]. Differences in V-ATPase structure of enzymes isolated from distinct tissues differing in the requirement for tonoplast energization was shown for *Ci. limon*. In the V-ATPase isolated from *Ci. limon* fruit tissue several additional 30–32 kDa polypeptides have been detected which are not present in the V-ATPase from *Ci. limon* hypocotyl tissue [51].

4.3. Structural changes of V_o

In addition to structural changes of the *Me. crystallinum* V-ATPase V_1-domain, CAM-related structural changes of the V_o-domain have been demonstrated. Freeze-fracture analyses revealed that intramembrane particles (IMPs) representing the V-ATPase V_o-domain exhibit a larger diameter in tonoplast

vesicles from plants in the CAM-state of photosynthesis compared to those in tonoplast vesicles isolated from plants in the C_3-state of photosynthesis [5,20]. Since on polyacrylamide gels the 16 kDa band representing subunit c is stained much heavier in CAM-tonoplast vesicles compared to C_3-tonoplast vesicles, it was suggested that the increase in IMP diameter is due to a higher number of copies of subunit c per V-ATPase holoenzyme [120]. An alternative explanation for differences in staining intensity and IMP diameter is the incorporation of different subunit c isoforms into V_o. A similar increase of V_o-diameter was observed during C_3-CAM shift in *Kalanchoë blossfeldiana* which is induced by short-day conditions, while salt treatment of *Ho. vulgare* plants did not affect V_o-size [109].

Variation in mineral nutrition seems to have an impact on V_o-structure. Differences in the V_o-domain diameter have been observed in tonoplast vesicles isolated from *Nicotiana tabacum* plants cultivated under *low* (2 mM) and *high* (20 mM) nitrate supply (M. Drobny, E. Fischer-Schliebs, personal communication). The V_o-domain of V-ATPases from *high*-nitrate plants turned out to be smaller than the V-ATPase V_o-domain of *low*-nitrate plants. Western blot analyses performed with two different antisera directed against the V-ATPase of *K. daigremontiana* revealed interesting results. While one antiserum cross-reacted with subunit c from *low*- and *high*-nitrate plants to a similar extent, indicating equal amounts of subunit c in both samples, a second antiserum strongly cross-reacted with subunit c of *high*-nitrate plants while subunit c from *low*-nitrate plants was poorly immunodecorated. Thus, one might suspect that distinct subunit c isoforms exhibiting differential cross-reaction with different antisera are expressed in *Ni. tabacum* plants under different nitrate supplies. In fact, it is intriguing that subunit c isoforms show differential immunological cross-reactions since subunit c is a highly conserved protein. On the other hand, differential immunological cross-reactions of V-ATPase c-subunits from different plant species have been previously reported [6]. Although the amino acid sequences of the *K. daigremontiana* and the *Ni. tabacum* subunit c differ only in three positions, the antiserum used in this study cross-reacted very well with the *K. daigremontiana* subunit c while there was almost no cross-reaction with the *Ni. tabacum* subunit c.

4.4. Possible impact of structural changes of V_o on the V-ATPase coupling ratio

As mentioned above (see Section 4.1), salt treatment of *He. annuus* [111] and *Ho. vulgare* [109] plants led to an increase in V-ATPase H^+-transport activity while ATP hydrolysis activity remained constant. In preparations from cultured cells of *D. carota*, H^+-transport activity increased while ATP-hydrolysis activity decreased [104]. This can be interpreted as a change in coupling ratio of both activities. In preparations from salt-treated *Me. crystallinum* plants both activities increased; however, this increase was higher for ATP-hydrolysis activity. Thus salt treatment also leads to a change of the coupling ratio of the V-ATPase in *Me. crystallinum*. In addition, in *Me. crystallinum* structural changes of the V_o-domain have been observed (see Section 4.3), which have been suggested to be due to a variation of the subunit composition of V_o. This modification of the V_o-domain might be correlated to changes in the coupling ratio of the V-ATPase. During salt treatment the salt includer *Me. crystallinum* accumulates high amounts of NaCl in the vacuole (the cell sap osmolality reaches values higher than 1 osmol kg^{-1}). Thus, after salt-induced CAM expression, nocturnal malate transport into the vacuole has to be performed against a high concentration gradient of solutes. Under such conditions a lower coupling ratio could be beneficial since the energy available from hydrolysis of ATP would be available for the transport of a lower number of protons. Activity measurements performed with *Me. crystallinum* tonoplast vesicles using the Palmgren [77] technique indicate that the coupling ratio of the CAM-V-ATPase indeed is lower than the coupling ratio of the C_3-V-ATPase (E. Berndt, R. Ratajczak, unpublished). The V_o-domain of the V-ATPase from *Ni. tabacum* grown under different nitrate supply exhibited similar structural changes as the V_o-domain of *Me. crystallinum*. In addition, the *high*-nitrate V-ATPase exhibits a lower coupling ratio compared to the *low*-nitrate V-ATPase (M. Drobny, E. Fischer-Schliebs, personal communication). On the other hand, in *Ho. vulgare* the salinity-induced change in coupling ratio of H^+-

transport and ATP hydrolysis was not correlated to visible structural changes of the V_o-domain [119]. Thus, it is difficult at this time to conclusively interpret the data mentioned above.

Since the structures of the V-ATPase and the F-ATPase are very similar one can suspect that modulation of the coupling ratio by variation of the number of proteolipids or by expression of different isoforms might also occur in the F-ATPase. In fact, there are hints that F-ATPase activity might be regulated in this way. As a response to changes in metabolic conditions (growth on glucose versus succinate) expression of F_o subunits of the *Escherichia coli* F-ATPase is modified leading to alterations in the subunit stoichiometry of the oligomeric F_o proton channel [121]. F-ATPases from mutants of the cyanobacterium *Synechocystis* containing proteolipids with different numbers of proton binding sites in the hydrophilic loop exhibit different coupling ratios (H.S. van Walraven, personal communication).

All plant subunit c isoforms cloned so far are homologues of the yeast *VMA3* gene which is essential for the production of an active V-ATPase holoenzyme [26]. However, in yeast two additional proteolipid genes have been identified [26], *VMA11* (coding for subunit c′) and *VMA16* (coding for subunit c″). The sequence similarity of *VMA11* and *VMA3* is 60%, while comparison of the *VMA16* sequence with sequences of *VMA3* and *VMA11* shows a similarity of ca. 30%. Vma3p and Vma11p contain four transmembrane helices, while in Vma16p five transmembrane helices are present [26]. It turned out that all three polypeptides are essential for the function of the V-ATPase holoenzyme. In plants, information about subunits c′ and c″ is poor. However, recently proteins exhibiting structural similarity to c″ have been identified in the worm *Caenorhabditis elegans* and *Ar. thaliana* by sequence comparison ([26,122]; M. Futai, personal communication). If c, c′ and c″ really are present in the plant V-ATPase V_o-domain, changes of coupling ratio of the V-ATPase might not only be due to the expression of different c-subunit isoforms (see above) but to the modification of the stoichiometry of c:c′:c″ in V_o. This might also explain the differential immunological cross-reactions of proteolipids from different plant species [6] and the results obtained with *Me. crystallinum*, *Ho. vulgare* and *Ni. tabacum* (see above).

5. Regulation of V-ATPase gene expression

As mentioned above, V-ATPase subunit message and protein amount is altered in response to environmental stress. Thus, it is likely that V-ATPase genes are highly regulated. A common feature of highly regulated plant proteins like the small subunit of ribulose-bis-phosphate carboxylase/oxygenase [123] or chlorophyll *a*/*b*-binding light-harvesting proteins [124] seems to be an endogenous oscillation of transcript amount. Such an endogenous oscillation has been demonstrated for the transcript amount of the *Me. crystallinum* V-ATPase subunit c [125]. Therefore, it can be suspected that the V-ATPase c-subunit and perhaps other V-ATPase subunits belong to the group of highly regulated proteins.

There are some hints that phytohormones might be involved in the regulation of V-ATPase gene expression and in the modification of the V-ATPase at the protein level. Treatment of *Ho. vulgare* plants with abscisic acid (ABA) significantly increased V-ATPase H^+-transport activity of tonoplast vesicles isolated from root tissue, while the H^+-transport activity was not affected by treatment with the cytokinin 6-benzyladenine [126]. In a subsequent study it turned out that in vivo treatment of *Ho. vulgare* with the P-ATPase inhibitor orthovanadate led to an increase in root internal ABA concentration and to an increase in ATP-dependent H^+-transport activity of tonoplast vesicles [127]. In *Me. crystallinum*, ABA treatment has been found to mimic salt-stress induced increase in message amount of a subunit c isoform [96]. On the contrary, in *L. esculentum* ABA does not seem to mediate NaCl-induced upregulation of the V-ATPase subunit A [100]. Thus, at this time it is difficult to say whether ABA plays a role in the regulation of V-ATPase genes or not. Indirect evidence for an involvement of the phytohormones ABA and ethylene in salt-stress response came from studies on *Citrus* seedlings, which exhibited increased levels of ABA and the ethylene precursor aminocyclopropane-1-carboxylic acid after salt shock [128]. Detailed information about the properties of promoters of V-ATPase genes would help to answer the question whether ABA or ethylene play a role in V-ATPase subunit expression. However, although genes encoding subunits of the V-ATPase have been cloned from many species (see Section 2

and Table 1), relatively little is known about the features of their promoters and how environmental stimuli modulate gene expression. For a long time the only information available about promoters of plant V-ATPase genes was a study on the promoter of the *D. carota* subunit A gene [129]. Recently, Lehr et al. [102] examined promoters from A- and c-subunit genes from *Be. vulgaris* and found higher promoter activity in the presence of NaCl, indicating that the salinity-induced increase in transcript amounts might be a result of transcriptional activation. Both promoters contain G-box motifs; however, it is unclear whether these G-boxes function in an ABA-mediated signal transduction pathway. In addition, the promoters exhibit CT-rich regions in the 5′UTRs. Similar CT-rich regions were found to be present in the promoter of the salt-inducible gene encoding for phosphoenolpyruvate-carboxylase [130] and might be structural elements involved in regulation of salinity-induced gene expression.

Beside the 'classical' plant hormones ABA and ethylene, jasmonic acid might play a role in V-ATPase modulation, at least at the protein level. Methyljasmonate treatment of *Ho. vulgare* leaves inducing senescence led to changes of the subunit pattern of the V-ATPase, to changes in pH-dependency of ATP-hydrolysis activity and to a decrease in inhibitor sensitivity [58].

The role of Ca^{2+} as a second messenger in biological systems is well established and changes in intracellular Ca^{2+} concentration could affect signal transduction pathways involving this divalent ion. Recently, Bressan et al. [131] pointed out the possible important role of Ca^{2+} for the regulation of V-ATPase expression. Liu and Zhu [132] were able to identify a possible candidate for a calcium sensing protein in *Ar. thaliana*: the protein encoded by the *SOS3* gene. Mutation of this gene leads to an increase in salt-sensitivity of the plant. SOS3 exhibits high sequence similarity to the yeast calcineurin B subunit and a neuronal Ca^{2+} sensor.

6. Intracellular distribution of the V-ATPase: one enzyme, many membranes?

Early fractionation experiments of membrane vesicles deriving from different cellular membrane systems indicated that the V-ATPase is not exclusively located at the tonoplast. V-ATPases were located in membrane vesicles deriving from the endoplasmic reticulum (ER) [133,134] and the Golgi apparatus [135–137]. Moreover, plasma-membrane enriched vesicle fractions obtained by sucrose density ultracentrifugation and/or two phase partitioning have been shown to possess V-ATPase-type activity and polypeptides [138–141]. However, from the data presented in the studies mentioned above it could not be excluded that the presence of V-ATPase in the ER, Golgi apparatus or plasma membrane fractions might be due to contamination from low amounts of tonoplast vesicles. More recently, studies on the intracellular distribution of V-ATPase by combination of biochemical and immunocytochemical techniques confirmed the finding that the V-ATPase is widely distributed within the endomembrane system including the ER, the Golgi apparatus, clathrin-coated vesicles and the plasma membrane [142–145]. Interestingly, the presence of V-ATPase in the plasma membrane seems to vary between different species. While in *Pi. sativum* cotelydon cells [146] the V-ATPase has been shown to be present at the plasma membrane, in cells of *Brassica oleracea* inflorescence [147], suspension-cultured tobacco BY-2 cells [148] and *Chlamydomonas reinhardtii* [149] it could not be detected at the cell surface by immunoelectron microscopy and V-ATPase subunits were absent in highly purified plasma membrane vesicles.

It is still an open question whether V-ATPases present in cellular compartments other than the tonoplast function as active H^+-pumps. An active V-ATPase has been reported to be present in vesicles deriving from the Golgi apparatus [135,136] and the ER [150]. In both cases the sensitivity to inhibitors of V-ATPase activity was lower compared to the tonoplast V-ATPase. Maeshima et al. [151] reported the existence of active V-ATPase in membrane vesicles from protein storage vacuoles of *Cucurbita* sp., acidifying the vesicle interior. The amount of V-ATPase present in the protein storage vacuoles increased during germination indicating de novo-synthesis of the enzyme and targeting to this special type of vacuole. Reports about active V-ATPases in the plant plasma membrane are contradictory. While the V-ATPase found in the plasma membrane isolated from *V. radiata* hypocotyl exhibited in vitro activity [139], the

plasma membrane located V-ATPases of *Pi. sativum* cotyledones [146] and of *Ricinus communis* seedlings and stem tissue ([145]; D.G. Robinson et al., unpublished) was inactive when assayed under conditions optimal for the tonoplast-located V-ATPase. In the latter examples, however, it cannot be excluded that cytosolic factors necessary for enzyme activity were lost during subcellular fractionation.

The wide intracellular distribution of the V-ATPase might be associated with membrane trafficking, i.e., the circuitous movement of membrane vesicles from the ER or Golgi apparatus to various cellular locations occurring within the plant cell, and it raises the question where the complex V-ATPase holoenzyme is assembled within the cell. From data obtained in studies of the yeast V-ATPase a model for the assembly of the V-ATPase holoenzyme was proposed, and information available for the plant V-ATPase suggest that the enzyme is assembled in the plant cell in a similar manner. In yeast it was shown that V_1 and V_o are independently synthesized [152]. There is evidence that subunits of V_o are cotranslationally inserted into the ER, where the intact V_o-domain is assembled [152,153]. For this assembly all V_o-subunits and, in addition, several ER proteins and chaperones are required. From investigation of plants hints for the involvement of the ER in vacuole formation came from electron microscopical studies performed during the reformation of vacuoles in evacuolated plant cell protoplasts [154]. Subsequent studies revealed that evacuolation led to the disappearance of the V-ATPase protein, which reappeared during the formation of a new vacuole [155,156]. The coupling of V_1-domains to V_o also seems to occur at the ER, either by attachment of complete V_1-domains assembled in the cytoplasm [153], or by subsequent attachment of V_1-subunits to V_o [157]. This is supported by immunocytochemical detection of subunit B at the ER of *Av. sativa* root cells [143] and immunological detection of V_o- and V_1-subunits in purified ER membrane vesicles isolated from *Z. mays* roots [144]. It is likely that the V_o–V_1 attachment requires the presence of ER proteins. By immunoprecipitation experiments Li et al. [158] were able to demonstrate close association of the V-ATPase with the ER resident chaperon calnexin and BiP (*bi*nding *p*rotein), which are good candidates to be involved in V-ATPase assembly.

Starting from the ER the assembled V-ATPase may enter the secretory pathway to be transported to its final destination in various intracellular membrane systems. As mentioned above the 95–110 kDa membrane integral subunit of V_o (homologue to the proteins encoded by the yeast *VPH1* and *STV1* genes) might be important for the correct targeting of the V-ATPase.

The intracellular distribution and the targeting of the V-ATPase might be even more complex since there is increasing evidence that there are subdomains of the ER and two biochemically distinguishable vacuolar compartments in the plant cell [159]. Hints for the existence of two functionally distinct vacuole types came from studies of tonoplast intrinsic proteins (TIPs) and of the V-ATPase. In *Pi. sativum* root tip cells α-TIP was found to be associated with vacuoles functioning in lectin storage, while TIP-Ma27 exclusively was detected in vacuoles containing the protease aleurain [160]. Mature motor cells of *Mimosa pudica* contain two distinct types of vacuoles, i.e., tannin vacuoles and aqueous vacuoles. Both γ-TIP and V-ATPase were mainly localized in the tonoplast of aqueous vacuoles [161]. Both examples raise the question how proteins like the V-ATPase or TIPs are targeted to one specific type of vacuole.

7. Future perspectives

Although the plant V-ATPase has been intensively studied, our knowledge about the regulation of its expression and activity in response to environmental and metabolic conditions has to be improved. At the gene expression level, information about characteristics of promoters of V-ATPase genes would be helpful to understand the involvement of signal transduction pathways in the regulation of V-ATPase genes. At the protein level there are some hints for regulatory mechanisms but more work is required to prove the hypotheses which can be drawn from the data available. An intriguing possibility of regulation is the modification of the subunit composition of the holoenzyme. This could be achieved by changes of the copy number of subunits in the holoenzyme complex or by incorporation of new subunits. In respect to the model of Junge et al. [19]

and the discovery of subunit c isoforms and the additional proteolipids c′ and c″, investigation of the V_o-domain seems to be promising to study the control of coupling ratio of the enzyme. To verify the role of structural modifications of V_o in the regulation of enzyme activity, more sequence information from plant V-ATPase proteolipid gene is required. Moreover, the V-ATPase has to be studied in plants grown under different environmental conditions in parallel with measurements of enzyme activity to correlate structure and function. Another open question is the role of V-ATPases found in different cellular membrane systems. Future experiments will be necessary to determine if these V-ATPases exhibit distinct subunit compositions which might be prerequisite for specific functions in different organelles.

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