Abstract
Two H\(^+\)-pumps may co-reside at the plant vacuolar membrane—the V-PPase and V-ATPase. Elucidation of their transport characteristics by patch clamp electrophysiology has indicated the possibility of distinct physiological roles. The V-ATPase may predominate in vacuolar acidification while the V-PPase may facilitate vacuolar K\(^+\) accumulation. Little is known of how pump activity is regulated in vivo, but an ATP\(^4^-\) regulated vacuolar shunt conductance may help clamp the membrane voltage to permit continued lumenal acidification. Both pumps respond to stress in order to maintain the critical functions of the vacuole. Under chill or hypoxic stress, V-PPase transcript levels and activity can increase to counter the impaired activity of the V-ATPase as ATP levels drop and the latter enzyme dissociates. During salt stress, the subunit composition of the V-ATPase may be modulated and its activity increased to power enhanced vacuolar Na\(^+\) sequestration.

Key words: V-ATPase, V-PPase, patch clamp, chill stress.

Introduction
When faced with a micrograph of a plant cell section, the eye is immediately drawn to those organelles with fine substructure. The mitochondria and chloroplasts bring to mind the relevant chapters of biochemistry texts, as structure is so closely identified with function. The same cannot be said of the vacuole despite the fact that its size may dominate the cell; it appears empty and so does not command immediate interest. However, despite its appearance, the vacuole is a multi-functional organelle involved in protein sorting, metabolite storage, signal transduction, and ionic homeostasis (Boller and Wiemken, 1986). Central to those physiological roles are the ion transport proteins of the membrane, among them two H\(^+\)-translocating pumps: the inorganic pyrophosphatase (V-PPase; EC 3.6.1.1) and the H\(^+\)-adenosine triphosphatase (V-ATPase; EC 3.6.1.3). Translocation of H\(^+\) from the cytosol to the vacuolar lumen acidifies the latter (typically to pH 5.5), providing optimal conditions for the hydrolytic enzymes vital to the organelle’s lysosomal function. Proton-pumping generates a transmembrane voltage (\(\Delta\psi\)) and pH gradient which together comprise a H\(^+\) electrochemical potential gradient (\(\Delta\mu\)H\(^+\)), the energy of which can be harnessed to drive H\(^+\)-coupled transport of solutes into the lumen. Thus the vacuole may act as a repository of, for example, sucrose, amino acids and inorganic solutes. But why is the vacuolar membrane endowed with two H\(^+\)-pumps? This question was posed a decade ago (Rea and Sanders, 1987) and an answer is slowly emerging, thanks to the cumulative efforts of several laboratories deploying techniques ranging from in vivo imaging to molecular biology and biophysics. Some of their results are brought together here in an assessment of how the pumps may function in vivo and of their roles in stress tolerance.

V-ATPase and vacuolar acidification
The V-ATPase is a large multi-meric H\(^+\)-pump comprising at least nine subunits (Sze et al., 1992), closely related to the F-type ATPases of energy-coupling membranes and occurring in plant, fungal and animal endomembranes. By conserving the free energy of cytosolic ATP hydrolysis in an H\(^+\) translocation step, its action in vivo is thought to facilitate vacuolar H\(^+\) accumulation. In plants, vacuolar pH (pH\(_v\)) is usually around 5.5, but has been observed to be below pH 3 in fruits or leaves which accumulate oxalic acid (Smith and Raven, 1976). That the V-ATPase can be critical to vacuolar acidification has
been demonstrated by gene disruption in *Saccharomyces cerevisiae*; a non-functional V-ATPase causes failure of luminal acidification and can be lethal depending on the external pH (Nelson and Nelson, 1990). Thus, the V-ATPase may be essential in cellular pH homeostasis.

To assess this pump's role in acidification and maintenance of cell pH requires an estimate of its operational transport coupling ratio \( n \)—the number of \( 
H^+ \) transported per ATP hydrolysed. Previous biochemical estimates of this key thermodynamic determinant of the pump's capacity for luminal acidification suggest a value of 2 \( \text{H}^+ \) per ATP (Bennett and Spanswick, 1984). Such results (from both plant and animal systems) have been derived from the comparison of initial rates of \( 
H^+ \) transport (using NMR or \( 
H^+ \)-sensitive fluorescent dyes) with ATP hydrolysis rates obtained from intact organelles, vesicles or the purified and reconstituted enzyme. However, this approach may be flawed by such uncertainties as membrane contamination, errors in buffering capacity estimates, uncoupling (where hydrolysis proceeds without concomitant transport), and passive \( 
H^+ \) leakage, all of which may distort the estimate of \( n \). To avoid these problems it is possible to exploit the electrogenicity of the V-ATPase using patch clamp electrophysiology, which affords a direct high resolution assay of V-ATPase activity in single intact vacuoles (isolated mechanically or by protoplast lysis).

Pump turnover is too low to resolve activity of a single protein and so turnover of the entire pump population of the membrane must be measured, afforded by the 'whole vacuole' patch clamp configuration (Hedrich and Neher, 1987). This configuration places the membrane and patch microelectrode in serial series (allowing experimental control of \( 
\Delta \phi \)) and gives complete control of the ionic conditions at both membrane faces. As the pump catalytic sites are exposed to the bathing medium, it is a simple matter to superpose the vacuole with ATP and measure V-ATPase activity as the macroscopic current generated as \( 
H^+ \) translocation is initiated. Use of this approach resulted in the first unequivocal demonstration of co-residence of both V-ATPase and V-PPase in a single vacuole (Hedrich *et al.*, 1989). To use the technique to determine \( n \) requires a consideration of the V-ATPase reaction:

\[
\text{ATP} + n[\text{H}^+]_c \rightleftharpoons \text{ADP} + \text{Pi} + n[\text{H}^+]_v \tag{1}
\]

where \( n \) cytosolic \( 
H^+ \) ([\( 
H^+ \])_c) are transported into the lumen at the expense of ATP (the forward or hydrolytic reaction) or \( n \) luminal \( 
H^+ \) ([\( 
H^+ \])_v) could be exported to the cytosol coupled to ATP synthesis (reverse or synthetic reaction). As with any enzyme reaction, an equilibrium position could be attained and for a pump this would manifest as zero net \( 
H^+ \) translocation (i.e. zero net current), with the pump poised between hydrolytic and synthetic modes. The \( \Delta \phi \) at which the pump would attain the equilibrium position is termed its reversal voltage \( (E_{\text{rev}}) \):

\[
E_{\text{rev}} = (RT/nF) \ln ([\text{ADP}][\text{Pi}][\text{H}^+]_v/K_{\text{ATP}}[\text{ATP}][\text{H}^+]_v)^* \tag{2}
\]

where \( n \) is the coupling ratio, \( K_{\text{ATP}} \) is the ATP hydrolysis equilibrium constant (which can be estimated for given ionic conditions), \([ ]\) denote activities and \( R, T \) and \( F \) have their usual meanings. Using patch clamping, it is possible to impose the transmembrane pH gradient of equation 2, activate the V-ATPase in both hydrolytic and synthetic modes by simultaneous superfusion with nucleotides and Pi and deduce a value of \( n \) from the measured \( E_{\text{rev}} \), values. To date this approach has only been applied to the V-ATPase of *Beta vulgaris* storage tissue vacuoles (Davies *et al.*, 1994), but has yielded unexpected results.

**V-ATPase coupling ratio determined by patch clamp**

Implicit in the application of equation 2 is the assumption that the V-ATPase is freely reversible under experimental conditions. This was demonstrated in the *Beta* study (Davies *et al.*, 1994) where the V-ATPase was activated by either ATP or ADP/Pi and the specific V-ATPase-mediated current (hydrolytic or synthetic, respectively) identified through use of a saturating concentration of bafilomycin A, as a specific and potent V-ATPase inhibitor (Bowman *et al.*, 1988). Such use of bafilomycin permits the pump current to be delineated more assuredly as interference from any other ATP-activated transporter is obviated. The drawback is that, in practice, it is not possible to wash out and obtain consecutive pump recordings under different conditions using the same vacuole. Despite this limitation, \( E_{\text{rev}} \) values have been obtained from *Beta* V-ATPase current/voltage (\( I/V \)) relationships, constructed by measuring the current generated at discrete clamping voltages by ATP/ADP/Pi first in the absence ('energized' \( I/V \)) then the presence of bafilomycin ('inhibition' \( I/V \)). Subtraction of the inhibition \( I/V \) from the energized yields the \( I/V \) difference relationship of the bafilomycin-sensitive (i.e. V-ATPase) current; the voltage at which no net current passes and, therefore, the pump is at equilibrium is by definition the pump's \( E_{\text{rev}} \). By using measured \( E_{\text{rev}} \) values to solve equation 2 for \( n \), it was found that, in contrast to kinetic estimates, \( n \) could apparently adopt variable and non-integer values, decreasing with increasing cytosolic pH (pH\(_c\)) at fixed vacuolar pH (pH\(_v\)) and increasing with increased pH\(_v\) at set pH\(_c\). For example, with set ATP/ADP/Pi concentrations of 5/5/10 mM, respectively, and pH\(_c\) set at 4.80, \( n \) decreased from 3.28 at pH\(_c\) 7.0 to 2.95 at pH\(_c\) 7.6.
Reaction kinetic modelling of V-ATPase activity

The phenomenon of high and variable coupling ratios is not new—values of $n$ of up to 10 have been reported for F-type ATPases (van Walvaren et al., 1986). At first sight, large coupling ratios may be taken to indicate equally (and perhaps unrealistically) large stoichiometries; that is, the number of $H^+$ binding/translocation sites of the enzyme (Laüger, 1991). However, this need not be the case and it is feasible that variable and non-integer values of $n$ could be generated by an enzyme which operates an incomplete coupling mechanism. In this event, the reaction cycle could proceed without full occupancy of all the available binding sites and the coupling ratio would effectively be less than the stoichiometry.

The $n$ values for Beta were deduced on the assumption of complete coupling of hydrolysis with transport in a single reaction loop. Expanding this scheme by the addition of a second loop (joined to the first by a reversible voltage-sensitive $H^+$ translocation step) with each loop having a discrete and integer stoichiometry for $H^+$ binding/translocation affords a reaction scheme which allows incomplete coupling. Such a model has been forwarded for the Beta V-ATPase (Davies et al., 1996) and relies on a maximum overall stoichiometry of 3 $H^+$, with the two linked cycles capable of binding one $H^+$ then (sequentially) a further two (Fig. 1). Occupancy of the $H^+$ binding sites at both cytosolic and lumenal enzyme faces would be determined simply by the prevailing pH. The voltage-sensitive reaction step linking the two cycles provides a 'choice' of routes for $H^+$ traversing the pump, resulting in the possibility of 1, 2 or 3 $H^+$ translocated per ATP. The observed values of $n$ would therefore be the manifestation of cycling of integer reactions, ultimately controlled by mass action effects of $H^+$. Application of this six-state model to V-ATPase $I/V$ relationships has given acceptable fits to data with a single set of rate constants, but only in the cases where pH$_c$ was fixed and pH$_v$ varied. This approach, therefore, appears promising, but now requires further development with the ultimate aim of relating model predications (such as binding site charge) to the known structure of the V-ATPase.

Physiological significance of the V-ATPase coupling ratio

As well as taking the reductionist approach to describe V-ATPase activity down to the last amino acid, the physiological implications of variable $n$ must be assessed. This can be done on a superficial level by estimating the theoretical maximum pH$_v$ that could be generated by the V-ATPase at a given pH$_c$. Re-arrangement of equation 2 to describe the $\Delta G$ of enzyme action,

$$\Delta G = nF\Delta \psi - RT\ln([\text{ADP}][\text{Pi}]/K_{\text{ATP}}[\text{ATP}])$$

and setting $\Delta G$ to zero allows pH$_v$ to be extracted for the relevant value of observed $n$ and observed in vivo values of nucleotides, Pi and $\Delta \psi$ (−20 to −50 mV; Bates et al., 1982; Spanswick and Williams, 1964). For example, $K_{\text{ATP}}$ has been estimated to be 6.48 x $10^5$ M at pH$_c$ 7.3 for a 'model' plant cell (Davies, 1997); at this pH the V-ATPase coupling ratio would be approximately 3. Taking [ATP], [ADP] and [Pi] as 2.3, 0.31 and 5 mM, respectively (Weiner et al., 1987; Rebeille et al., 1984) and assuming a constant $\Delta \psi$ of −20 mV, pH$_v$ could decrease to approximately 4.7, bringing the pump to equilibrium. This adequately accounts for typical plant and fungal pH$_v$. Severe vacuolar acidification by the V-ATPase would necessitate a decrease in coupling ratio—the lowest recorded was 1.75 (obtained at pH$_c$ 8.0) which substituted into equation 3 (with the appropriate change to $K_{\text{ATP}}$) yields a maximum pH$_v$ of 2.88 at $\Delta \psi = -20$ mV. This value would account for all but the most acidic of fruits and leaves. Critically, there is no evidence as yet which describes an acidic vacuole with such alkaline cytosol and...
so there must remain a question mark over the use of the observed Beta coupling ratios in this context.

However, the calculations lend some weight to the proposal that the V-ATPase of profoundly acidic compartments such as lemon juice-sac vacuoles most probably operates close to equilibrium to maintain pH below 3 (Müller et al., 1996). As the latter authors have stressed, the interesting question arising from playing with $\Delta G$ calculations is 'what factors prevent the V-ATPase from over-acidifying the lumen?'. Unfortunately, the V-ATPase of juice-sac vacuoles is insensitive to bafilomycin in its native membrane (Müller et al., 1996) which precludes a reliable patch clamp evaluation of the effects of pH on the coupling ratio of this isoform. By comparing transport properties of vacuolar vesicles from lemon juice-sacs (in vivo pH, 6.5 declining to 2.2 during maturation) with seedling epicotyls, Müller et al. (1996) highlighted H$^+$ permeability of the membrane and differential sensitivities of the V-ATPase isoforms to oxidation and pH as possible regulatory mechanisms.

Applicable to possibly all V-ATPase isoforms is regulation of activity by $\Delta \psi$. If, for the sake of argument, the calculations given above are re-worked for $\Delta \psi = -50$ mV, then the pH$_v$ maxima become 5.16 at pH$_h$ 7.3 and 3.39 at pH$_h$ 8.0. V-ATPase activity would increase the magnitude of $\Delta \psi$, with the lumenal build-up of positive charge eventually restricting pump activity and limiting acidification. Dissipation of charge to clamp $\Delta \psi$ could be achieved by a shunt conductance; e.g. the entry of Cl$^-$ into the lumen or exit of positive charge to the cytosol, mediated by a channel protein (Fig. 2).

**ATP$^4^-$-regulated shunt conductance**

The majority of animal endomembranes energised by a V-ATPase so far examined contain a Cl$^-$-conducting shunt to help regulate $\Delta \psi$ (Al-Awqati et al., 1992). However, there are precedents for counter-ion conductances which permit K$^+$ translocation in lysosomes and endocytic vesicles (Galloway et al., 1983; Harikumar and Reeves, 1983). In yeast vacuoles, two Cl$^-$ transporters have been identified (with differential affinities for Cl$^-$ and inhibitor sensitivities) which could promote vacuolar acidification (Wada et al., 1992). Increasing luminal Cl$^-$ has been suggested to activate an anion-selective channel in Beta vacuoles (Plant et al., 1994), which would facilitate enhanced anionic influx to the lumen.

To operate effectively, the activities of a pump and its shunt must, in some way, be regulated mutually. For example, phosphorylation is known to increase activity of the V-ATPase in macrophages (Nordstrom et al., 1994) and of putative animal endomembrane Cl$^-$ shunt conductances (Bae and Verkman, 1990). The obvious mutual regulator is $\Delta \psi$ itself, but it is feasible that the moieties which control pump action (such as pH, nucleotides and Pi) could modulate channel activity to achieve the desired effect of promoting the V-ATPase hydrolytic mode.

A macroscopic conductance in Beta vacuolar membrane has been shown to respond to V-ATPase substrates (Davies et al., 1995). This so-called 'instantaneous' current (i.e. it lacks an obvious time-dependency and can manifest macroscopically immediately in response to the application of voltage) was first characterized by Hedrich and Neher (1987) as a predominantly cationic conductance, operative at low (less than 1 $\mu$M) cytosolic Ca$^{2+}$. In the absence of divalent cations, addition of ATP (with ATP$^4^-$ as the predominant species) to the cytosolic face of Beta vacuoles in the whole vacuole configuration induced a significant increase in the macroscopic instantaneous current throughout a ±100 mV voltage range (Davies et al., 1995). Dissection of the I/V relationships revealed an essentially linear ATP-dependent current (insensitive to bafilomycin) which reversed close to the equilibrium voltage of the dominant monovalent cation in the experimental system (K$^+$ or Na$^+$), strongly suggesting that the induced current was carried by that ion. Relative permeability ratios of the ATP$^4^-$-activated current ($P_K: P_{Cl}: P_{Na}: P_{Cl}$ 4:1) supported the conductance as being largely cationic.

With K$^+$ concentration symmetrical across the Beta membrane or with an approximately 3-fold greater K$^+$ concentration in the lumen than the extravacuolar face, ATP$^4^-$ activated an inwardly-directed conductance over the physiological $\Delta \psi$ range of 0 to 50 mV (i.e. movement of K$^+$ out from the lumen). In addition, cytosolic Mg$^{2+}$ and pH increased the instantaneous current, with pH probably not only acting directly but also

![Fig. 2. Membrane voltage is a determinant of maximal vacuolar acidification by the V-ATPase. At $\Delta \psi = -50$ mV, the predicted maximum pH$_v$ is only 5.16 at pH$_h$ 7.3. If the ATP$^4^-$-regulated shunt conductance allows partial dissipation of the transmembrane K$^+$ gradient, $\Delta \psi$ would reduce. At $\Delta \psi = -20$ mV, maximum pH$_v$ would be 4.7.](image-url)
indirectly through effects on speciation of ATP (Davies et al., 1995). This instantaneous current could be increased by a putative in vivo concentration of ATP$^{4-}$ (0.15 mM) in the additional presence of an in vivo concentration of the V-ATPase substrate MgATP$^{2-}$ (baflomycin was present to stop interference by the pump).

Taken in total, it would appear that this conductance could operate in vivo in conjunction with the V-ATPase, both being regulated by relative concentrations of activating ligands. As plant vacuoles accumulate K$^+$ under nutrient-replete conditions there is a strong driving force for passive K$^+$ release from lumen to cytosol. Such a cycle would be rendered less futile if K$^+$ release were to be the shunt conductance of the V-ATPase. The ATP$^{4-}$-activated conductance appears to fulfill the requirements of that role, but it is one which it may not occupy alone and former links between co-regulation of Cl$^-$ conductance and V-ATPase activity in vacuoles are awaited.

**V-PPase and vacuolar K$^+$ accumulation**

As stated previously, plant vacuoles exhibit a marked capacity to accumulate K$^+$. Luminal K$^+$ ([K$^+$]$_v$) varies with cell type and K$^+$ availability, but is typically around 200 mM under replete conditions (Leigh and Wyn Jones, 1984; Malone et al., 1991). Cytosolic K$^+$ ([K$^+$]$_c$) is thought to be maintained homeostatically around 80–100 mM (Leigh and Wyn Jones, 1984; Maathuis and Sanders, 1994). Transport of K$^+$ into the lumen against concentration and electrical gradients would therefore require energization. This could be achieved by coupling K$^+$ uptake to $\Delta$μH$^+$ and indeed, a K$^+$-H$^+$ antiporter activity has been described for *Brassica napus* hypocotyl vesicles (Cooper et al., 1991), but its activity is inhibited at K$^+$ concentrations over 25 mM.

The complete dependence on K$^+$ of V-PPase hydrolytic and H$^+$-pumping activities has led to the proposal that K$^+$ not only stimulates the enzyme but is also a translocated ion, rendering the V-PPase a novel K$^+$ pump. Patch clamp studies of the Beta V-PPase (Davies et al., 1991) demonstrated the vectorial dependence on K$^+$ of electroneutrality, consistent with the V-PPase acting as a direct K$^+$-H$^+$ symport (i.e. both ions are translocated in the same direction). Pyrophosphatase could only activate the hydrolytic pump mode when K$^+$ was present at the cytosolic enzyme face (independent of [K$^+$]$_v$) and orthophosphatase only activated the synthetic mode in the presence of luminal K$^+$ (independent of [K$^+$]$_v$).

Evidence supporting K$^+$ translocation has thus far only come from the patch clamp approach. Equation 2 can be expanded to encompass putative K$^+$ transport with H$^+$ by the V-PPase thus;

$$E_{rev} = RT/(n + m)F \left( \frac{[Pi]^2[H^+]_c[K^+]_c^n}{K_{PP}[PPi][H^+]_c^2[K^+]_c^m} \right)$$

where $m$ is the coupling ratio for K$^+$. The $E_{rev}$ of a (Pi/PP)-dependent V-PPase current depends not only on the trans-membrane H$^+$ gradient but also that of K$^+$. The $E_{rev}$ values of the V-PPase current have been found to respond to changes in both ionic gradients in a manner consistent with the predictions arising from equation 4 for the behaviour of a symport pump (Davies et al., 1992). The deduced coupling ratio from the data was 1.3 H$^+$/1.7 K$^+$/Pi which in vivo would poise the V-PPase in hydrolytic mode, allowing vacuolar K$^+$ accumulation.

At present there are insufficient data to support either a thorough examination of coupling ratio or reaction kinetic modelling of the I/V relationships. Moreover, independent confirmation of direct K$^+$ translocation by the V-PPase has not been forthcoming. In part this may be due to the technical approaches available; vacuolar vesicles have a high K$^+$ permeability and the high $K_m$ for K$^+$ (40 mM; Rea et al., 1996) is at odds with the micromolar concentration required to support a significant intravesicular accumulation ratio (Rea et al., 1996). The enzyme's implicit electrogenic may also foil attempts to detect intravesicular K$^+$ accumulation. Using vacuolar vesicles of *Vitis vinifera* loaded with fluorescent probes Ros et al. (1995) failed to detect PPi-driven transport. However, the resultant $\Delta \phi$ in that study of over 200 mV inside-positive would be sufficient to drive K$^+$ out of the lumen passively.

If the V-PPase were to act in vivo as a K$^+$-H$^+$ symport pump, then the tentative 1.3 H$^+$/1.7 K$^+$ coupling ratio would allow a theoretical maximum [K$^+$]$_v$ of approximately 670 mM to be attained at $\Delta \phi = -20$ mV, $p_\text{H} = 7.3$ and $p_\text{H} = 5.5$. This would explain even the [K$^+$]$_v$ values for vacuoles of open stomata (500 mM). However, operation of the V-PPase would be restricted by both $\Delta \phi$ and the extreme values of $p_\text{H}$. The increased $p_\text{H}$ generated by the V-ATPase. As the magnitude of $\Delta \phi$ increases and $p_\text{H}$ decreases, there would come a point at which the V-PPase would be reversed and translocate K$^+$ into the cytosol with concomitant synthesis of Pi (Davies, 1997). Such a premise would effectively see the vacuole engaging two K$^+$ conductances (V-PPase and ATP$^{4-}$-activated instantaneous current) with the possibility of severe dissipation of the K$^+$ gradient.

It is possible that the V-PPase is under strict kinetic control in vivo (e.g. by Ca$^{2+}$; Rea et al., 1992), but another solution to the problem of pH$_i$-induced V-PPase reversal is the temporal separation of the two pumps. In pumpkin cotyledons, V-PPase activity decreases with ageing whilst V-ATPase activity increases (Suzuki and Kasamo, 1993). Immunofluorescent microscopy of *Triticum* leaves has shown a reciprocal abundance of the two pumps during development (Darley, Marrison, Leach, Rea, Sanders, and Davies, unpublished data). In developing tissue (characterized by a preponderance of pro-vacuoles) the V-PPase is the predominant pump,
where it may facilitate vacuolar K⁺ accumulation to drive cell expansion. At maturity the V-ATPase is by far the most abundant. Future studies which seek to explore pump abundance and concomitant pHₐ/K⁺, may throw further light on the inter-relationships between the V-ATPase and V-PPase.

**Lashings of stress**

**Chilling and hypoxic stress**

The central question as to why plants have two vacuolar H⁺-pumps can usefully be posed in the context of stress physiology. During the changes in respiratory states which accompany hypoxia and chill stress, cellular PPI levels tend to remain stable whilst those of ATP decline (Dancer and ap Rees, 1989; Weiner et al., 1987). The kinetic limitation placed on the V-ATPase under these conditions could be exacerbated further during chill stress by cold-induced dissociation of its catalytic subunits (Moriyama and Nelson, 1988; Yoshida et al., 1989). Such dysfunction may manifest itself as a drop in cytoplasmic pH during the early stages of stress imposition (Yoshida, 1994). The possibility that during V-ATPase restriction the V-PPase may function as an auxiliary ‘fail-safe’ pump to prevent irrevocable cellular damage by effectively capitalizing upon stable PPI levels is supported in some part by the examination of ∆Gₐₜₚ and ∆Gₚₚ values.

By estimating concentrations of cytoplasmic ATP and PPI complexes using published values of relevant species from non-photosynthesizing cells, it has been possible to describe the changes in predicted free energies of ATP and PPI hydrolysis which might occur during hypoxic- or cold-induced cytoplasmic acidosis (Davies et al., 1993). Under normal conditions, the ∆Gₐₜₚ and ∆Gₚₚ of this model cell would be —51 and —27 kJ mol⁻¹ respectively at pHₙ 7.3 and with an [ATP]/[ADP] ratio of 7.42. For simulated anaerobiosis at pHₙ 7.3 with the [ATP]/[ADP] ratio dropping to an arbitrary value of 0.24, ∆Gₚₚ remains unaffected, but ∆Gₐₜₚ decreases to —42 kJ mol⁻¹. If the adverse nucleotide ratio were accompanied by acidosis to pHₙ 6.0, ∆Gₐₜₚ would become —41 kJ mol⁻¹, but ∆Gₚₚ would *increase* to —36 kJ mol⁻¹, indicating that PPI would be a more favourable phosphoryl energy donor under stress conditions.

Treatment of *Acer pseudoplatanus* cells with respiratory inhibitors was shown to decrease significantly cellular ATP levels, but only halve cellular PPI (Macri et al., 1995). A vacuolar pH gradient was still maintained during respiratory blockade, but could be reduced by inhibiting the V-PPase with imidodiphosphate, supporting the premise that the latter pump could compensate for V-ATPase failure. Also it would appear that as in chill-stressed or oxygen-deprived rice, V-PPase transcript levels increase 10-fold (Carystinos et al., 1995), the plant’s survival strategy may not simply be a passive reliance on favourable PPI energetics.

Further evidence for the importance of the V-PPase in maintaining the pH gradient across the vacuolar membrane has come from a study on chill-stress of cold-sensitive mung bean hypocotyls (Darley et al., 1995). Imposition of low temperature (4°C) on 7-d-old hypocotyls for 72 h reversibly arrested growth, allowing test plants to be treated as being at the same developmental stage as control plants harvested at 7 d. Chilling produced a marked and sustained increase in extractable alcohol dehydrogenase (ADH) activity, indicating the onset of fermentative metabolism in agreement with previous findings of chill-induced *de novo* ADH synthesis in maize and rice (Christie et al., 1991).

Both hydrolytic and H⁺-pumping V-PPase capacities (measured using vacuolar-enriched vesicles) increased 2-fold over control levels during the first 48 h of the stress regime (Darley et al., 1995). In contrast, V-ATPase activities and passive H⁺ permeability remained virtually unaffected. The differential between V-PPase and V-ATPase activities was maintained when assays were performed at either 37°C or 4°C. It seems likely that the enhanced performance of the V-PPase was due to its increased abundance rather than an increase in turnover. Quantifying changes in immunoblot signal on probing vesicular protein with antibodies raised against the V-PPase and 65 kDa V-ATPase subunit showed again a 2-fold increase in V-PPase content, but unaltered V-ATPase. That V-ATPase activity did not decline during chill stress (when it would be expected that the enzyme would dissociate) is most likely the result of the non-freezing temperature used.

Although it is clear that the V-PPase does respond to chill stress, its precise temporal behaviour during the critical early stages of a stress challenge which results in reduced ATP levels and acidosis remains unclear. The chill-induced 2-fold increase in V-PPase polypeptide would hardly compensate for the 85–90% decrease in activity known to occur when mung bean V-PPase is assayed at 4°C rather than 24°C (Yoshida and Matsuura-Endo, 1991). Rather it is feasible that the up-regulation of the enzyme is of greatest consequence in the recovery of pH homeostasis as the induced stress is lifted.

The factors which trigger changes in the abundance of the V-PPase in stress responses have not been identified. However, ABA is thought to promote anaerobiosis tolerance (Hwang and Van-Toai, 1991) and treatment of barley roots with ABA can significantly increase the activities of both H⁺-pumps, again with an increase in V-PPase polypeptide (Kasai et al., 1993). Both cold acclimation and ABA application cause accumulation of transcripts which hybridize to BN59, a cDNA clone isolated from winter *Brassica napus* showing homology to the sequence encoding the 70 kDa V-ATPase nucleo-
tide-binding subunit (Orr et al., 1995). The physiological significance of that result has not been demonstrated, but it suggests a responsive capacity for the V-ATPase in cold survival.

Salt stress

The responses of the vacuolar H^+ pumps to salt stress vary with plant and cell type, developmental stage, and severity and duration of stress imposition. Formation of a cohesive physiological story is by no means easy when the dynamics and implications of responses have to be considered in the context of an imperfect understanding of spatial and temporal differences in pump action under normal conditions.

Imposition of salt stress on mung bean seedling root tips results in the elevation of pH, from 5.6 to 6.2 within 3 h, if external Ca^{2+} is low (Nakamura et al., 1992a, b). In this system, the V-PPase may be the predominant pump. Nakamura et al. (1992a, b) have proposed that low external Ca^{2+} permits elevation of cytoplasmic Na^+ (by influx through the plasma membrane), which in turn inhibits V-PPase activity resulting in lumenal alkalization. Relatively little is known of long-term responses of the V-PPase. Salt-adapted Acer suspension cells show greater activities for both pumps over non-adapted, but with a preferential increase in the V-PPase (Zingarelli et al., 1994).

The V-ATPase exhibits complex responses to salt stress. The amount of pump protein may decrease (Nicotiana; Reuveni et al., 1990), increase or subunit preponderance may alter (Bafluls et al., 1995). In Citrus leaf cells, salinity induces no change in V-ATPase specific activity in relation to total protein, but the structure of the hydrophilic head (V_1 sector) changes, possibly due to proteolytic degradation (Bafluls et al., 1995). In leaf tissue of the CAM plant Mesembryanthemum, NaCl (but not osmotic stress) induces increased activity and increased abundance of the c subunit of the integral pore-forming V_0 sector (Tsiantis et al., 1996). The significance of changes in V-ATPase activity may lie in the finding of a concomitant increase in the activity of the vacuolar Na^+-H^+ antiporter in Mesembryanthemum, which suggests that the activities of both systems increase to permit enhanced vacuolar Na^+ sequestration (Tsiantis et al., 1996).

Changes in the V-ATPase may be linked to developmental status; NaCl stress of Lycopersicon produces increased levels in mRNA for the 70 kDa subunit in expanded but not unexpanded leaves (Binzel, 1995). The response is transient, with a return to control levels within 7 d, indicating that putative up-regulation is an acute survival strategy. In contrast, increased abundance of subunit c occurs in both mature and juvenile tissue of Mesembryanthemum (Tsiantis et al., 1996). The signalling events which precede modulation of subunit abundance have not been identified. ABA has been implicated in the c subunit Mesembryanthemum response as its exogenous application can mimic the effects of salt stress (Tsiantis et al., 1996). However, Binzel and Dunlap (1995) elegantly demonstrated the independence of NaCl-induced changes in the 70 kDa subunit message from changes in endogenous ABA in Lycopersicon. Regardless of the induction pathway, little is as yet known of the subtle consequences in V-ATPase action which alteration in subunits might bring.

Summary

Vacuolar H^+-pumps are complex entities and their importance to the plant cell under normal or stress conditions should not be overlooked. It is vital that technically a multi-disciplinary approach should be maintained in elucidating their function. In particular, emphasis should be placed on characterizing the action of specialist isoforms as it is clear that data arising from ‘model’ systems such as Beta cannot yield definitive answers. Also, studies on the pathways which control developmental and stress-induced changes in pump abundance and substructure are now urgently required.

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