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# Vacuolar transporters and their essential role in plant metabolism

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## Abstract

**Following the unequivocal demonstration that plants contain at least two types of vacuoles, scientists studying this organelle have realized that the plant 'vacuome' is far more complex than they expected. Some fully developed cells contain at least two large vacuoles, with different functions. Remarkably, even a single vacuole may be subdivided and fulfil several functions, which are supported in part by the vacuolar membrane transport systems. Recent studies, including proteomic analyses for several plant species, have revealed the tonoplast transporters and their involvement in the nitrogen storage, salinity tolerance, heavy metal homeostasis, calcium signalling, guard cell movements, and the cellular pH homeostasis. It is clear that vacuolar transporters are an integrated part of a complex cellular network that enables a plant to react properly to changing environmental conditions, to save nutrients and energy in times of plenty, and to maintain optimal metabolic conditions in the cytosol. An overview is given of the main features of the transporters present in the tonoplast of plant cells in terms of their function, regulation, and relationships with the microheterogeneity of the vacuome.**

Key words: Channel, energization, localization, membrane, storage, tonoplast, transport, transporter, vacuole.

## Introduction: divide et impera

In this review it will be shown how vacuolar transporters are an integrated part of a complex cellular network, enabling the plant to react properly to changing environmental conditions, store nutrients and energy in time of plentiful supply, and maintain optimal metabolic conditions in the cytosol.

Following the unequivocal demonstration that plants contain at least two types of vacuoles, storage and lytic (Paris *et al*., 1996; Jauh *et al*., 1999), it has become clear that the plant 'vacuome' is far more complex than expected. Vacuoles can be classified according to their soluble proteins, and by the class of aquaporins (for a review, see Luu and Maurel, 2005) present in their membranes. Jauh *et al*. (1999) showed that the tonoplast of storage vacuoles contains δ-TIP (tonoplast intrinsic protein), while the tonoplast of protein storage vacuoles containing seed-type storage proteins are marked by α-TIP and δ-TIP, and sometimes with γ-TIP as well, whereas the tonoplasts of vacuoles storing vegetative storage proteins and pigments contain δ-TIP and γ-TIP. Vacuoles marked only with γ-TIP have the characteristics of lytic vacuoles.

It appears that vacuoles are organelles which fulfil highly specialized functions depending on tissue and cell type, and their developmental stage. All vacuoles seem to contain the vacuolar-type $H^+$-ATPase (V-ATPase; Sze *et al*., 1999), $H^+$-translocating inorganic pyrophosphatase (V-PPase; Maeshima, 2000, 2001; Drozdowicz and Rea, 2001), and TIP-like aquaporins (King *et al*., 2004; Luu and Maurel, 2005) that differ in their function depending on the type of vacuole in which they reside.

The function of the vacuole differs depending on whether its role is, for example, a protein storage vacuole in a seed, a storage vacuole in a root tip, or a vacuole in a fully developed leaf cell (Otegui *et al*., 2002). However, the vacuole system is even more complex. Some fully

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developed cells contain two or more large vacuoles which exhibit different functions, as it has been shown that even in a single cell distinct types of vacuoles may occur. This is the case for *Mimosa pudica* motor cells, which contain one vacuole which stores large amounts of tannins, and a second vacuole lacking these compounds, but exhibiting a far higher density of aquaporins and $H^+$-ATPase, indicating that this compartment is mainly responsible for the rapid loss of water during movement of pulvini (Fleurat-Lessard *et al*., 1997).

*Mesembryanthemum crystallinum* is a plant able to change its photosynthetic metabolism from $C_3$ to crassulacean acid metabolism (CAM) if exposed to drought or salt stress. Epimashko *et al*. (2004) showed that leaf cells of *Mesembryanthemum* plants induced to perform CAM by NaCl treatment contained two large vacuoles. One is used to accumulate NaCl, and the second to store malic acid during the night. Remarkably, even a single vacuole may also be subdivided and fulfil several functions. This has been shown for protein storage vacuoles, where internal membranes separate storage proteins from both phytic acid and proteins that are characteristic of lytic vacuoles (Jiang *et al*., 2001).

Another emerging topic is the flow of materials between different vacuoles, and how large and small vacuoles present in one cell communicate and share certain functions (Marty, 1999; Reisen *et al*., 2005). It is tempting to speculate that compounds which impair plant metabolism will be removed first by small vacuoles that have a higher surface-to-volume ratio. The difference in surface-to-volume ratio does not change the final theoretical accumulation rate, but it allows a fast and efficient removal of potentially disruptive metabolic compounds from the cytosol. However, since the volume of these small vacuoles is limited, a large storage capacity requires their final transfer to the larger central vacuole.

These considerations indicate that the vacuole is much more than an organelle which simply expands to allow cell expansion or acts as a rubbish dump for the cell, as is often mentioned in older textbooks. The appropriate vacuole in the right place is a prerequisite for a plant to survive in response to environmental constraints, to meet future changing demands, and to optimize the cellular metabolism. Unfortunately, the description of all these aspects is beyond the scope of this review which will be limited to a discussion of the role of the large central vacuole and the contributions of the tonoplast transporters that have been identified and described at the molecular level so far.

The large central vacuole, which is best characterized in leaf cells, serves a number of functions. (i) It enables cells to reach a large size and, in leaves, it allows chloroplasts to be distributed for optimal light capture and efficiency. (ii) It is a store for nutrients that may be used in future growth phases or for seed development. Plants develop new leaves only if they sense that the nutritional conditions permit further growth. The temporary storage of nutrients within the vacuole allows them to be accumulated in preparation for the next step in growth and development. (iii) The metabolism of a plant must be tightly controlled. The vacuole allows the cell to keep the cytosolic concentrations of ions and metabolites optimal for metabolism. (iv) As stated in old textbooks, the vacuole is also the rubbish dump of a cell. However, even this may be simplistic because compounds formerly thought to be waste products are now thought to contribute to plant fitness, for example, as anti-herbivory agents.

A number of published reviews have been devoted to the vacuole and vacuolar transport processes. These previous reviews give more information about the identification of transporters and the biochemical characteristics of carrier and channel proteins (Boller and Weimkem, 1986; Matile, 1987; Martinoia, 1992, Martinoia *et al*., 2000, 2002; Leigh and Sanders, 1997; Robinson and Rogers, 2000; Maeshima, 2001). Here, the aim is to give a general overview, linking transport processes to cellular metabolism and highlighting the impact vacuolar transporters exert to allow optimal function, and to increase plant fitness.

## Functions require energy

Due to the large volume of the central vacuole, a compound which is present at similar concentrations in the cytosol and in the vacuole would be present in ~5-fold higher amounts within the vacuole. However, the concentration of a large number of compounds in the vacuolar lumen exceeds the corresponding concentrations in the cytosol by several fold, hence a chemical and/or electrical gradient is required to allow this accumulation. This process requires energy, and two proton pumps are responsible for the necessary driving forces, namely a V-type ATPase and a V-PPase (Rea and Sanders, 1987; Sze *et al*., 1999; Maeshima, 2000; Kluge *et al*., 2003). As far as we know, all types of vacuoles possess both types of these proton pumps (Hedrich *et al*., 1989). A point which should be mentioned is that while acidification of the vacuole can reach <3–4 pH units, the membrane potential difference ($\Delta\Psi$) between the vacuolar lumen and the cytosol is always reported to be rather low (~30 mV). This observation indicates that anion fluxes play a major role in dissipating the membrane potential generated by the pumps and thus keeping an equilibrum between the chemical and electrical components of the electrochemical membrane potential difference for protons.

### *The V-ATPase*

The V-ATPase is universally present in the membranes of different internal acidic organelles in eukaryotic cells. In addition, this type of proton pump has been found on the plasma membrane of both insects and some bacterial species. The V-ATPase has an intricate structure which is

related to the F-type ATPases. Basically, two functional parts can be distinguished: a peripheral $V_1$ sector which contains three copies of the A- and B-subunits, responsible for the catalytic activity, and the subunits C–H which form a central stalk linking the $V_1$ to the hydrophobic, membrane-embedded $V_0$ sector (Sze *et al*., 2002). The $V_0$ sector contains the a-subunit and six copies of the c-subunit which forms a proton-conducting channel and arose from gene duplication and fusion of the 8 kDa c-subunit of the $F_0$ sector from F-type ATPases. As in their F-type homologues, where ATP is regenerated by induced conformational changes due to a rotatary mechanism, parts of the V-ATPases have been shown to rotate when ATP is supplied, suggesting a very similar enzymatic mechanism for both proton pumps (Imamura *et al*., 2003; Makyio *et al*., 2005).

In plants, transcriptional regulation is an important mechanism for adjusting V-ATPase activity. For example, expression of subunits is up-regulated under salt stress (Maeshima, 2001; Kluge *et al*., 2003, and references therein). Much less is known about metabolic regulation of V-ATPase activity, but it is likely that the redox state of the plant cell influences it (Tavakoli *et al*., 2001). The recent demonstration that a WNK kinase can phosphorylate subunit C indicates that post-translational modifications are also involved in the regulation of the V-ATPase (Hong-Hermesdorf *et al*., 2006).

### *The V-PPase*

The second vacuolar proton pump uses PPi as its energy source. This compound is synthesized in many metabolic reactions, such as DNA and RNA synthesis, sucrose and cellulose synthesis, or the conversion of pyruvate to phosphoenolpyruvate (PEP) by pyruvate phosphate dikinase. In contrast to the V-ATPase, the V-PPase consists of a single polypeptide (Rea *et al*., 1992; Maeshima, 2000). It is found in plants, algae, photosynthetic bacteria, protozoa, and archaebacteria, but not in fungi or mammals. In plants, two isoforms are often found, a potassium-dependent isoform and a potassium-independent isoform (Belogurov and Lahti, 2002).

Generally, V-PPase activity is high in young tissues. However, in some cases, such as in grape berries, the V-PPase is also the predominant vacuolar proton pump in mature plant cells. Grape berries are very acidic, and it is astonishing that in a tissue where vacuoles are highly acidic (pH <3) the V-PPase is the predominant pump (Terrier *et al*., 1998). This may be due to the fact that grape berries are exposed to the sun and consequently reach high temperatures. Since the V-PPase is constituted by a single polypeptide, it may be less subject to degradation compared with the V-ATPase. In line with this hypothesis is the observation that grape berry V-PPase is heat stable, and exhibits a temperature optimum of ~50 °C.

Overexpressing the vacuolar $H^+$-PPase AVP1 in *Arabidopsis thaliana* resulted in plants exhibiting a higher salt tolerance, which was probably a consequence of an increased proton gradient across the tonoplast (Gaxiola *et al*., 2001). A more detailed analysis of these *Arabidopsis* mutants, and corresponding plants where the expression of *AVP1* was down-regulated, revealed that altering *AVP1* expression resulted in aberrant plant development (Li *et al*., 2005). Interestingly, not only was the vacuolar pH affected in mutants mis-expressing *AVP1*, but so was the apoplastic pH. Plants lacking AVP1 exhibited a significantly more alkaline apoplastic and vacuolar pH, whereas *AVP1*-overexpressing plants exhibited a more acidic apoplastic pH value (no data shown for the vacuole). Despite the fact that in plants overexpressing AVP1 some V-PPase protein was localized in the plasma membrane, this observation is most probably due to a concomitant pleiotropic regulation of plasma membrane $H^+$-ATPase activity. These results are consistent with the assumption that pH gradients across the tonoplast, and across the plasma membrane, are somehow interconnected and form a regulatory network.

### *Consequences of building up an electrochemical gradient*

The $\Delta$pH and $\Delta\Psi$ generated by the V-ATPase and V-PPase can be used to transport compounds against their concentration or electrochemical potential gradients (Fig. 1). As will be described in more detail below, $Na^+$, $Ca^{2+}$, and several heavy metals are accumulated within the vacuole using proton antiport mechanisms. The $\Delta$pH is the driving force, and the stoichiometry between the compound imported and the proton exported defines the theoretical maximal accumulation of a specific cation. Anions are accumulated by the inside-positive $\Delta\Psi$, which has been reported to be ~30 mV (Martinoia *et al*., 2000).

However, it is still unclear whether, and by how much, $\Delta\Psi$ varies in response to environmental conditions or metabolic changes. According to the Nernst equation, a $\Delta\Psi$ of 30 mV (inside positive) would allow a ~5-fold vacuolar accumulation of a monovalent anion such as $Cl^-$ or $NO_3^-$ relative to the concetration in the cytosol, and a 10-fold accumulation of a divalent anion such as $SO_4^{2-}$. In the case of carboxylates, the more acidic conditions within the vacuole may lead to a change in the compound's protonation state and, consequently, a so-called acid trap mechanism may operate if the transporter involved is specific for one form of the carboxylate. For some inorganic anions such as nitrate, higher degrees of accumulation than a factor of five have been postulated (Martinoia *et al*., 1981). In this case a proton–anion antiport mechanism would allow a higher accumulation, even if its operation would be highly electrogenic due to movement of positively and negatively charged ions in opposite directions.

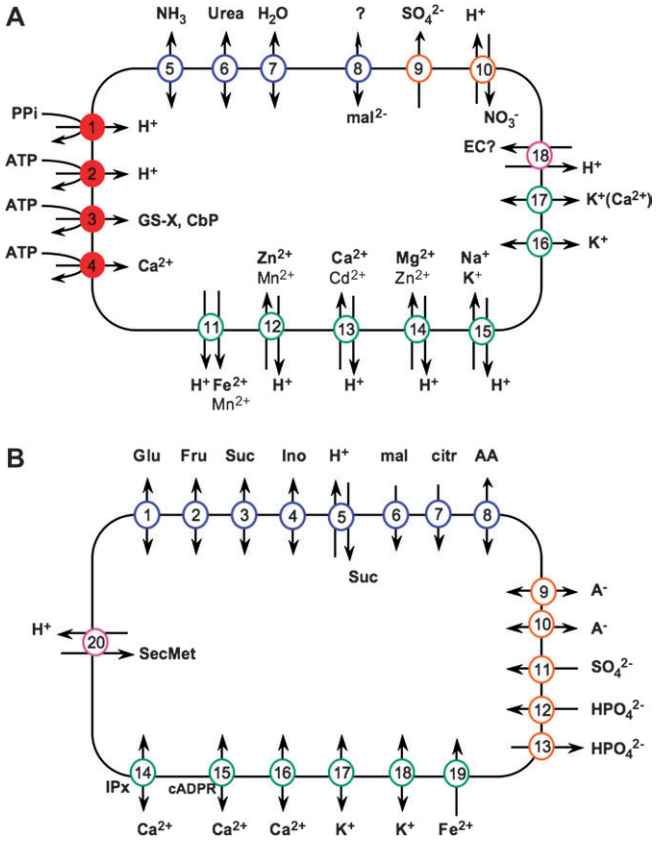


**Fig. 1.** Vacuolar transporters described at the molecular level (A) and postulated from electrophysiological data, transport experiments, and compartmentation studies (B). (A) Red, directly energized transporters; blue, transporters for water and organic solutes; orange, inorganic anion channels and transporters; green, cation transporters; pink, secondary metabolite transporters. 1, V-PPase; 2, V-ATPase; 3, MRP-type ABC transporter; 4, P-type $Ca^{2+}$ pump; 5–7, tonoplastic intrinsic proteins (TIPs) are permeable for water, urea, and ammonia; 8, malate transporter AttDT. 9, sulphate exporters; 10, nitrate proton antiporter; 11, iron exporter NRAMPs; 12, zinc (and at least in some plants $Mn^{2+}$) transporters MTPs; 13, CAX proteins transport mainly $Ca^{2+}$ but may also transport other divalent cations such as $Cd^{2+}$; 14, magnesium transporter, probably also transports zinc (Shaul *et al.*, 1999; not described in the text); 15, NHX transporters can accumulate both $Na^+$ and $K^+$ within the vacuole; 16, the TPK1 (KCO1) channel may correspond to the VK channel; 17, the SV channel corresponds to TPC1, 18, MATE transporter, genetic evidence indicates that this is an epicatechin transporter. Note that for genes which form small families (MTP, NRAMP, NHX, and CAX), not all members have been localized so far and some of them may therefore be located on other membranes. (B) From transport experiments and compartmentation studies, it must be postulated that transporters for glucose, fructose, sucrose, and inositol exist (1–5). Depending on the vacuole type, hexoses and sucrose are transported either by facilitated diffusion or by a proton antiport mechanism (for a detailed discussion, see Martinoia *et al.*, 2000). Recent data (Hurth *et al.*, 2005) indicate that in addition to the described malate transporter, a malate/fumarate channel as well as a citrate transporter are present in the vacuolar membrane (6, 7). Transport experiments revealed the presence of probably two amino acid transporters (8), a chloride and nitrate (or general anion) channel (9, 10), a sulphate importer (11), and a phosphate transporter (12); a phosphate exporter must also be postulated (13). Several calcium channels have been described: an IPx-gated $Ca^{2+}$ channel, a cADPRibose-activated $Ca^{2+}$ channel and a voltage-gated calcium channel (14–16). In addition to the recently identified SV channel, electrophysiological data show the presence of the FV potassium channel, which might correspond to the VK channel (Bihler *et al.*, 2005) and the FV potassium channel (17, 18). For more details about $Ca^{2+}$ and $K^+$ channels, see the text and the cited literature. An $Fe^{2+}$ uptake transporter must be postulated (19). Many secondary metabolites have been localized within the vacuole, but knowledge about the proteins involved in their transport across the tonoplast is scarce (20).

In addition to transport processes energized by the electrochemical gradient, the transport of some compounds is directly energized. $Ca^{2+}$ can be transported into the vacuole by a P-type $Ca^{2+}$ pump (Geisler *et al.*, 2000*b*), and large organic anions such as glutathione conjugates or chlorophyll catabolites are transported by ABC-type transporters (Martinoia *et al.*, 1993; Lu *et al.*, 1998; Tommasini *et al.*, 1998) (see below for more details). Taking published values for cytosolic ATP, ADP, and Pi concentrations, it can be calculated that such a transport allows a theoretical accumulation ratio of $\sim 10^8$ (Kreuz *et al.*, 1996).

## Save in times of plenty, spend in times of need

In natural ecosystems, nutrients such as nitrogen and phosphorus are often limiting factors for plant growth. In general, plant growth depends on both the availability of carbohydrates produced during photosynthesis and the accumulation of macro- and micronutrients. Roots are able to proliferate in nutrient-rich soil patches, and to extract nutrients which, if in excess of immediate needs, can be stored for subsequent growth if the external supply falls. With this simple strategy, a plant can cope with soils having only a few nutrient-rich patches, or with changes in climatic conditions provoking either a decreased nutrient uptake (e.g. due to drought) or leaching of nutrients from the soil (e.g. during a period of heavy rain).

### *The role of the vacuole in nitrogen nutrition*

It was demonstrated in the early 1980s that nitrate accumulates within the central vacuole of leaf cells (Martinoia *et al.*, 1981). Later studies examined whether nitrate concentrations in the cytosol are kept constant or fluctuate. Siddiqi and Glass (2002) provided evidence for nitrate homeostasis, while Britto and Kronzucker (2003) argued in favour of a variable nitrate pool. In a very recent report, Radcliffe *et al.* (2005) presented convincing evidence that nitrate in maize root tip cells increased from a few millimolar to 13 mM when the cells were exposed to 10 mM $NO_3^-$. Concomitantly, the vacuolar nitrate concentration increased from 20 mM to ~80 mM. However, these authors mentioned that this increase of vacuolar nitrate concentration might be restricted to the rapidly expanding root tip cells, because they could not detect such changes in fully expanded root cells located more distantly from the root tip. These observations imply that knowledge obtained for vacuoles from one type of tissue cannot necessarily be transferred to vacuoles from other tissues.

Classical experiments using an indirect assay of $H^+$ transport provided evidence for an $NO_3^-/H^+$ antiporter in the tonoplast (Blumwald and Poole, 1985; Schumaker and Sze, 1987). In a search for the function of putative anion channels, Geelen *et al*. (2000) isolated a deletion mutant for the *Arabidopsis AtCLC*-a channel. Homozygous deletion mutants exhibited normal development, and no obvious phenotypic alteration (Geelen *et al*., 2000). However, when these plants were analysed for anion contents, the deletion mutants showed a reduced capacity to store $NO_3^-$, while chloride, sulphate, and phosphate levels were not affected. In addition, chlorate, which is reduced within the cell by nitrate reductase and acts as a herbicide, induced a more pronounced chlorosis in mutant plants. It was found that the *AtCLC*-a gene was expressed in roots and shoots, and its transcript levels increased after supplying nitrate (Geelen *et al*., 2000). Although the subcellular localization of this putative channel protein remains to be shown, the authors postulated that *AtCLC*-a might play a critical role in controlling intracellular nitrate status.

Bearing in mind that the $\Delta\Psi$ between the cytosol and the vacuole is not sufficient to drive the accumulation observed for nitrate, a vacuolar nitrate channel would not explain the *in vivo* compartmentation, and the earlier transport data. However, very recently, a completely new picture of CLC channels has emerged. Accardi and Miller (2004) and Piccolo and Pusch (2005) demonstrated that a homologue to the prokayotic $Cl^-$ channel (CLC) and mammalian ClC acts as a chloride/$H^+$ antiporter. Indeed, in a very recent study by De Angeli *et al*. (2006) evidence is presented that AtCLCa is localized in the vacuolar membrane and, using electrophysiological methods, the authors could demonstrate that this CLC channel behaves as an $NO_3^-/H^+$ exchanger allowing the accumulation of nitrate within the vacuole.

Beside nitrate, ammonia ($NH_4^+$ and $NH_3$) and urea are major storage forms for nitrogen (Fig. 1). Despite the fact that ammonia is potentially toxic because it acts as an uncoupler, millimolar concentrations have been reported to occur in plants. Ammonia is taken up as $NH_4^+$ into the cell by ammonia transporters (AMTs) which are present in almost all organisms (von Wirén and Merrick, 2004). The driving force is the electrochemical potential difference across the plasma membrane. Within the cytosol, an equilibrium between $NH_4^+$ and $NH_3$ is established, leading to ~3% of the $NH_3$ form at a cytosolic pH of 7.7.

Recently it was shown that two aquaporins, AtTIP2;1 and AtTIP2;3, containing a hydrophilic histidine residue, instead of a typical hydrophobic aromatic residue in the narrowest pore constriction region, are permeable to $NH_3$ (Loqué *et al*., 2005). As for water transport, lipid bilayers are fully permeable to $NH_3$, but specific transport proteins are required to reduce cytosolic ammonia contents efficiently. Within the vacuole, a new equilibrium between $NH_3$ and $NH_4^+$ is established, leading to a trapping of ammonia as $NH_4^+$. Since the vacuolar pH is about two units more acidic when compared with the cytosol, total ammonia concentrations will be ~100-fold higher in the vacuole. Thus, assuming a total cellular ammonia concentration of 2 mM, most of which will be in the vacuole, the cytosolic concentration of this potentially toxic compound will be only 20 μM. If required for amino acid and protein synthesis, utilization of cytosolic ammonia will build up a chemical gradient towards the cytosol, and ammonia can be released from the vacuole.

Most higher plants develop severe symptoms of toxicity if exposed to high ammonia concentrations. Resistance mechanisms decreasing ammonia uptake have been shown to be present at the root level. While roots of barley, which is an ammonium-sensitive plant, do not down-regulate ammonium uptake and consequently require a large amount of energy to remove $NH_4^+$ taken up from the soil, rice roots maintain the ammonia equilibrium at an energetically neutral, near-Nernstian value (Britto *et al*., 2001).

It is tempting to speculate that the vacuolar pH is another factor affecting ammonia tolerance, since vacuolar trapping of ammonia is a direct function of the ΔpH between the cytosol and the vacuole. Despite these considerations, it should be mentioned that not all members of the AMT family have been localized and so some may contribute to ammonia detoxification. However, if an AMT is localized in the tonoplast, a proton antiport mechanism has to be postulated. A transporter dependent only on the voltage difference would not allow accumulation, but would exclude $NH_4^+$ from the vacuole, and it is well documented that weak bases such as methylamine and neutral red are accumulated within the vacuole. This effect would not occur if an $NH_4^+$ uniport existed.

Urea is the major nitrogen form supplied in fertilizers in agriculture, and is taken up by urea transporters located at the plasma membrane of roots (Liu *et al*., 2003). Investigations on human aquaporins have shown that, besides water, some members are able to transport urea (Ishibashi *et al*., 1997). These data led to a reinvestigation of transport properties of plant aquaporins, and it has been demonstrated that two tobacco aquaporins, the plasma membrane *Nt*PIP1 and the tonoplast *Nt*TIPa, mediate urea fluxes (Gerbeau *et al*., 1999). Further studies showed that heterologous expression of various *Arabidopsis* vacuolar aquaporins (*At*TIP1;1, *At*TIP1;2, *At*TIP2;1, and *At*TIP4;1) conferred growth to a urea-defective yeast mutant and facilitated urea transport when expressed in oocytes (Liu *et al*., 2003). *AtTIP1;1*, *AtTIP2;1*, and *AtTIP4* were up-regulated during early germination and under nitrogen deficiency, supporting their role in nitrogen nutrition (Liu *et al*., 2003).

### *The vacuole as an intermediary store for sulphate*

Sulphate and phosphate have also been shown to accumulate in vacuoles (for a review, see Martinoia *et al*.,

2000). It is interesting to note that newly emerging leaves of some plants such as *Macroptilium atropurpureum* showed symptoms of sulphate starvation although mature leaves were found to contain appreciable quantities of sulphate (Bell *et al*., 1994). Tracer exchange experiments revealed that the rate constant for the exchange of vacuolar ($k_v$) $SO_4^{2-}$ is slow compared with that of the cytosol ($k_c$), indicating that under $SO_4^{2-}$ starvation, the cytosolic pool is depleted faster than the vacuolar pool. This observation may indicate that some plants are not adapted to sulphur starvation and that not only vacuolar uptake, but also release of nutrients and metabolites is essential for plant growth and development.

In this context, it is important to mention that Kataoka *et al*. (2004) identified two distinct vacuolar localized putative sulphate transporters. SULTR4-1 is highly expressed in roots even in the presence of high sulphate concentrations. Under sulphur starvation, SULTR4-1 expression is induced in shoots and SULTR4-2 is induced in both shoots and roots. Vacuoles isolated from the *sultr4;1*/*sultr4;2* double knock-out mutants contained higher amounts of sulphate than those of wild-type plants. At the whole plant level, much higher sulphate contents could be found in roots of the *sultr4;1*/*sultr4;2* double knock-out mutants, whereas the sulphate contents in the shoots remained constant. These results strongly support the hypothesis that these two sulphate transporters are responsible for the efflux of sulphate from the vacuole into the cytosol. It would be interesting to investigate the metabolic consequences of these mutants by analysing the impact of these mutations on enzymes implicated in sulphur assimilation and also to know whether these plants are hypersensitive to heavy metals since these chelate to thiol-containing compounds generated by sulphate reduction. Remarkably, no tonoplast transporter mediating sulphur uptake into the vacuole has been described so far.

## Keep your cell clean, but don't be the rubbish dump

As sessile organisms, plants have to cope with their environment. Transporters at the root plasma membrane are generally not sufficiently specific to exclude structurally similar, but undesired compounds. For example, it is known that transporters allowing the uptake of some micronutrients, such as iron or zinc, also transport cadmium, which is highly toxic. Furthermore, potentially toxic compounds of hydrophilic structure may enter a cell by diffusion and can be harmful if the cell does not possess suitable detoxification mechanisms. Moreover, even at leaf level, plants are not able to exclude undesired compounds. For example, hydrophobic air pollutants may slowly diffuse through the cuticle or, as their hydrophilic counterparts, enter the leaf through widely opened stomata.

### *The vacuole plays a central role in avoiding salt stress*

Worldwide, a large part of agricultural soil has to be irrigated. Part of this water is taken up by plants and another part evaporates, while only minor amounts of the minerals dissolved in the water, mostly NaCl, are taken up by the plant. Consequently, irrigation leads to soil salinity. Salinity is therefore not only a problem for plants living in ecological niches, but has become a serious problem in modern agriculture.

Much effort has been devoted in the last years to identify factors which confer salt resistance. Besides the genes identified in the SOS system (Zhu, 2002), such as a plasma membrane $Na^+/H^+$ exchanger (SOS1), a protein kinase (SOS2), and a calcium-binding protein (SOS3), the vacuolar $Na^+/H^+$ antiporter is one of the most important factors implicated in NaCl tolerance (Fig. 1). The first vacuolar $Na^+/H^+$ antiporter was identified in *Saccharomyces cerevisiae* (Nass *et al*., 1997), and an A*rabidopsis* $Na^+/H^+$ antiporter (*At*NHX1) was identified by sequence homology (Apse *et al*., 1999; Gaxiola *et al*., 1999). Overexpression of *AtNHX1* in *Arabidopsis* resulted in plants exhibiting increased salt tolerance (Apse *et al*., 1999).

This result was extremely interesting from an agricultural standpoint but raised some basic questions about cell physiology. It must be assumed that vacuolar accumulation of $Na^+$ is mainly a function of the pH difference between the cytosol and the vacuolar lumen. Increasing $Na^+$ tolerance by overexpressing an $Na^+/H^+$ antiporter would mean that either efflux of vacuolar $Na^+$ is so fast that in a wild-type cell the $Na^+$ retrieval system is insufficient to confer salt tolerance, or that by overexpressing the $Na^+/H^+$ antiporter some cellular parameter, such as the vacuolar $Na^+$ efflux, is changed, the $\Delta$pH is increased, or vesicle formation and trafficking which might be responsible for rapid $Na^+$ uptake may be altered. Surprisingly, a recent DNA array analysis, comparing *Arabidopsis* wild type and *At*NHX1 deletion mutants (Sottosanto *et al*., 2004), showed that even in the absence of NaCl many transcript levels are altered. Mainly genes related to transcription and cell signalling exhibited altered expression levels. Interestingly, the gene set exhibiting altered transcription levels under salt stress was different from the set observed under control conditions. It must therefore be concluded that *At*NHX1, in addition to its role as an $Na^+/H^+$ antiporter, plays a significant role in intracellular vesicular trafficking and transcription regulation.

These results indicate that *At*NHX1 is also part of a complex network, and are in line with the observation that the vacuolar $Na^+/H^+$ exchange activity is dependent on the presence of both SOS1 and SOS2 (Qiu *et al*., 2004). In *sos1* deletion mutants, $Na^+/H^+$ activity is significantly higher, while in *sos2* deletion mutants this activity is strongly reduced. Activated SOS2 protein added *in vitro* increased tonoplast $Na^+/H^+$ exchange activity in vacuolar vesicles

isolated from mutants lacking SOS2, but had no effect on vacuolar $Na^+/H^+$ activity of wild-type plants, or *sos1* or *sos3* deletion mutants. These results indicate that the vacuolar $Na^+/H^+$ activity is a target of the SOS pathway, and that there may be a co-ordinated regulation of the tonoplast and plasma membrane sodium exchange.

Bearing this in mind, it would be interesting to investigate the expression of the SOS pathway in *Arabidopsis* lines overexpressing *At*NHX1. Deletion mutants have shown that the function of *At*NHX1 is not restricted to salt tolerance (Apse *et al*., 2003). When grown under control conditions, mutants lacking *At*NHX1 had reduced leaf area and a reduction in the frequency of large epidermal cells. Since *At*NHX1 is also a $K^+/H^+$ antiporter, the phenotype observed for the *At*NHX1 T-DNA insertional mutant may indicate that these plants are no longer able to supply the vacuole with sufficient amounts of potassium for the normal expansion of leaf cells. Such a change in leaf expansion would obviously have implications on overall cellular metabolism.

*Arabidopsis* harbours six *At*NHX isoforms, and for five of them $Na^+/H^+$ transport activity has been demonstrated (Yokoi *et al*., 2002; Aharon *et al*., 2003). *AtNHX1* and *AtNHX2* are the most highly expressed members of this family, and corresponding transcripts are widely distributed, while *AtNHX3* and *AtNHX4* transcripts are almost exclusively present in flowers and roots. A topological analysis has revealed that the C-terminus of *At*NHX1 is located in the vacuolar lumen (Yamaguchi *et al*., 2003), and deletion of this C-terminus results in a substantial increase in $Na^+/H^+$ transport activity. In addition, the $Na^+/K^+$ ratio was twice that of wild-type plants.

In a subsequent study, Yamaguchi *et al*. (2005) presented evidence that this C-terminal region can interact with a vacuolar calmodulin-like protein in a $Ca^{2+}$- and pH-dependent manner. These results are highly interesting, since they provide evidence that a tonoplast transporter might be regulated by changing conditions in the vacuolar lumen. However, information on changes in vacuolar pH and $Ca^{2+}$ is scarce. If the regulation is based on the presence of the calmodulin-like protein, then it must be assumed that this is a rather long-term regulation, since most probably *de novo* protein synthesis is required.

Accumulation of $Na^+$ or $K^+$ in vacuoles may affect vacuolar pH, as is the case for the anthocyanin-containing petal cells of morning glory, in which NHX1 is specifically expressed during flower opening (Yamaguchi *et al*., 2001; Yoshida *et al*., 2005). Accumulation of $K^+$ (or $Na^+$) results in alkalization of the vacuole and an increase in the osmotic pressure of cells during petal opening. The authors suggested that this may be the driving force for cell enlargement and colour shift leading to flower opening and the famous blue colour.

Very recently, Nakamura *et al*. (2006) described the vacuolar localization of rice *Os*ClC1 and *Os*ClC2 channels. Heterologous expression of these putative channels partially complemented the yeast *gef1* mutant, which lacks the sole yeast ClC channel. *OsClC1* was up-regulated during salinity. This channel is therefore likely to be the counterpart to NHX for the accumulation of $Cl^-$. Whether it works as a channel, as suggested by the authors, or as a $Cl^-/H^+$ antiporter, which would be able to accumulate at a high ratio of chloride within the vacuole, remains to be determined.

### *The vacuole is an integrated part of heavy metal homeostasis and detoxification*

Heavy metals often pollute soils, either because they contain a naturally high amount of these compounds or because of human activities. Most progress on the role of the vacuole in heavy metal transport, homeostasis, and detoxification has been made with the micronutrients zinc and iron. Despite the fact that these heavy metals are required by the plant in several organelles, it is a prerequisite that a plant maintains low concentrations of them in the cytosol. The cytosol must not evacuate all zinc or iron, but the concentrations of the free metals must be kept extremely low to avoid toxic symptoms. Thus, these elements must be released into the cytosol in a highly controlled way to supply enzymatic functions.

Presently, very little information is available concerning intracellular iron movement in plants (Briat, 2006). The bulk of iron in leaves is found within the chloroplasts where it is engaged in photosynthetic processes. Plastids contain ferritin, an iron storage protein, and iron-ferritin represents >90% of the iron found in a pea embryo axis (Briat *et al*., 1999; Briat, 2006).

Plant vacuoles are likely to play a major role in accumulating iron present in excess, and releasing iron into the cytosol, if required by metabolism. An observation which is in favour of such a hypothesis is that upon iron overload, or in pea mutants overaccumulating iron, nicotianamine concentrations rise, and the bulk of this iron chelator is found within the vacuole, whereas most nicotianamine is present in the cytoplasm under normal conditions or during iron deficiency (Pich *et al*., 2001).

In yeast, vacuolar iron uptake and release have been studied in detail and it has been shown that the vacuole serves as an internal iron reservoir. Iron is transported into the yeast vacuole by a carrier named CCC1p and is likely to be stored as a ferric hydroxide in the lumen, which is less reductive compared with the cytosol. Recycling very probably involves a reductive step by the enzyme Fre6p, and finally iron can be released via the Smf3p and Fet5p transporters (Kwok and Kosman, 2006). In plants, no vacuolar iron uptake system has been described so far. However, databank searches have revealed that a gene highly homologous to CCC1p exists in plants and might act as a vacuolar $Fe^{2+}$ transporter (E Martinoia and E Neuhaus, unpublished data). Further candidates are gene products of

the YSL family. In maize, as in other grasses, iron is taken up as a complex with mugineic acids (phytosiderophores). The transporter catalysing this uptake has been identified and called YS1 (yellow stripe), due to the phenotype of maize lacking this transporter (Curie *et al*., 2001). Despite the fact that *Arabidopsis* roots do not take up iron as $Fe^{3+}$-mugineic acid, but as $Fe^{2+}$, this model plant contains seven YS-like (YSL) genes. The observation that nicotianamine, a precursor of the phytosiderophores, has been found in vacuoles of plants accumulating high levels of iron within suggests that YSLs could serve as vacuolar iron importers.

A third possibility is that an ABC-type transporter facilitates iron–nicotianamine transport into the vacuole, since such a complex has some characteristics resembling phytochelatin–Cd complexes (Clemens *et al*., 1999; Vatamaniuk *et al*., 1999), but in the case of nicotianamine, the complex is formed between the nitrogen donor centres and carboxyl groups of nicotianamine and the heavy metal, and not between the thiol residues and the heavy metal, as in the case of phytochelatins.

More is known about vacuolar iron export. A gene family called NRAMP has been shown to be involved in bacterial heavy metal transport (Gunshin *et al*., 1997; Agranov *et al*., 1999; Chen *et al*., 1999; Thomine *et al*., 2000). Heterologous expression of *AtNRAMP3* in yeast revealed that this transporter mediates iron transport (Thomine *et al*., 2000, 2003). Furthermore, this study showed that *AtNRAMP3* transcript levels were up-regulated upon iron starvation. Further studies revealed that plants lacking *AtNRAMP* did not show any phenotype when grown under Fe-sufficient conditions, but, upon Fe starvation, the plants contained increased amounts of Zn and Mn. Overexpression of *AtNRAMP* resulted in the opposite effect. Interestingly, overexpression of *AtNRAMP* provoked a down-regulation of the primary Fe uptake system, IRT1 (Henriques *et al*., 2002; Vert *et al*., 2002). The latter result again demonstrates that the expression and activity of transporters residing in different membranes and tissues are interconnected within a complex network. It is tempting to speculate that both decreased and increased AtNRAMP activity results in changes in cytosolic iron pools that can be sensed and used to modulate the transcription of transporters and enzymes involved in iron assimilation.

The final proof of *At*NRAMP3 function was presented recently by producing a double knock-out *Arabidopsis* plant *atnramp3*/*atnramp4* (Lanquar *et al*., 2005). Like *At*NRAMP3, *At*NRAMP4 also resides in the vacuolar membrane. In the *atnramp3*/*atnramp4* double knock-out mutant, seed germination is arrested under low Fe nutrition, despite the fact that mutant seeds showed wild-type Fe levels. These data indicate that *At*NRAMP3 and *At*NRAMP4 are required to mobilize Fe from the vacuolar globoids of seed cells, and very probably also from other tissues. Whether *At*NRAMPs also play an important role in the release of manganese and zinc remains to be established.

Zinc is required as cofactor for >1200 transcription factors and enzymes in *Arabidopsis* (Krämer and Clemens, 2006*b*), and plants usually require between 15 μg and 300 μg of zinc $g^{-1}$ of dry mass (Marschner, 1995). However, if too much zinc is present in the cytosol, it may displace other ions such as $Fe^{2+}$ and $Mn^{2+}$ from their respective binding sites. Several ZIP transporters are implicated in zinc uptake at the plasma membrane (Krämer and Clemens, 2006*a*). In order to limit free cytosolic zinc, plants can either down-regulate zinc uptake systems, increase compounds that complex zinc, or transfer zinc present in excess in the cytosol into the vacuole.

The cloning of ZAT (zinc transporter of *A. thaliana*) provided the first candidate for a vacuolar zinc transporter (van der Zaal *et al*., 1999). This transporter was later renamed MTP1 (metal tolerance protein; Mäser *et al*., 2001), and is a member of the cation diffusion facilitators (CDF) protein family (Krämer and Clemens, 2006*a*). Ectopic overexpression of *MTP1* resulted in plants that exhibited a slightly greater zinc accumulation, and improved growth under supraoptimal zinc concentrations, than wild-type plants. From these results, the authors concluded that MTP1 could be a vacuolar zinc transporter.

Further studies in other laboratories support this hypothesis. It was shown that proteoliposomes harbouring reconstituted MTP1 protein could accumulate zinc (Bloss *et al*., 2002), and subcellular localization studies confirmed the presence of MTP1 in the vacuolar membrane (Kobae *et al*., 2004). Blaudez *et al*. (2003) expressed the poplar *PtdMTP1* ectopically in *Arabidopsis* and observed increased zinc tolerance in the transgenic plants. MTP1 also confers some tolerance to Co. Interestingly, the *Thlaspi goesingense* MTP1 has been reported to confer tolerance to a broad spectrum of heavy metals including Ni, Cd, and Co (Persans *et al*., 2001). Kobae *et al*. (2004) isolated a mutant lacking *At*MTP1 and observed that it was more sensitive to increased zinc levels in the medium, confirming that *At*MTP1 is an important factor in keeping the cytosol free of zinc excess. The fact that transcript levels of *At*MTP1 do not change in response to altered zinc concentrations argues that *AtMTP1* is a housekeeping gene.

Since *A. thaliana* contains in total eight MTP1 homologues (http://aramemnon.botanik.uni-koeln.de/), it is tempting to speculate that other members of this family might also play a role in vacuolar zinc transport, either under specific conditions or in individual tissues. It is interesting to note that in the zinc-hyperaccumulating species *Arabidopsis halleri*, *MTP1* expression is constitutively high (Dräger, 2004), and that in an interspecies cross between *A. halleri* and *A. lyrata*, *MTP1* loci co-segregate with zinc tolerance (Dräger, 2004). An MTP from *Stylosanthes* (*Sh*MTP1), a plant growing on acid soils and accumulating large amounts of zinc, has been identified and expressed heterologously in yeast and *Arabidopsis*. It was demonstrated that *Sh*MTP1 is targeted to the

vacuolar membrane, and confers manganese tolerance (Delhaize *et al*., 2003). It is still unknown which transporter might release zinc from the vacuole under zinc-limiting conditions. A possible candidate might be one of the NRAMPs.

In *S. cerevisiae*, two members of the CDF family, Zrc1p and Cot1p, are both localized on the vacuolar membrane, and confer resistance to zinc when overexpressed and sensitivity when deleted (Regalla and Lyons, 2006). Zrt3p is closely related to the $Zn^{2+}$ uptake transporter ZupT from *Escherichia coli*, and is involved in the release of $Zn^{2+}$ from vacuolar stores.

An intriguing observation is that NRAMPs, MTPs, and CAXs (see below) all have a broad substrate spectrum. It will be an ambitious goal for future research to determine the respective contribution of the different transporters to the movement of divalent cations across the tonoplast. However, the fact that some of the corresponding knock-out plants exhibit pronounced phenotypes suggests that the different transporters probably have well-defined roles in plants.

The vacuole also plays an important role in cadmium detoxification (Rea *et al*., 1998; Martinoia *et al*., 2000). Several transporters such as MTPs, CAXs (see below), and ABC transporters may act as cadmium or cadmium chelate transporters. In contrast to *S. cerevisae* (Li *et al*., 1997) and *Schizosaccharomyces pombe* (Ortiz *et al*., 1995), where vacuolar-localized ABC transporters have been demonstrated to play a central role in cadmium detoxification, no plant vacuolar ABC transporter has been identified that transports cadmium–glutathione or cadmium–phytochelatin complexes. However, ectopic expression of the yeast ABC transporter YCF1 in *Arabidopsis thaliana* improved cadmium tolerance, arguing that the vacuolar cadmium transport activity is a limiting factor in cadmium resistance (Song *et al*., 2003).

### *Secondary compounds and xenobiotics*

The vacuole is also the classical store for secondary plant compounds and modified xenobiotics. Since several review articles have been published on this topic in the recent past (Kreuz *et al*., 1996; Rea *et al*., 1998; Theodoulou, 2000; Martinoia *et al*., 2000, 2005; Yazaki, 2005; Klein *et al*., 2006), this fascinating role of plant vacuoles is not reviewed here extensively but a few recent studies are merely mentioned.

In barley, endogenously produced flavonoid glucosides, such as isovitexin and saponarin, are transported by a proton antiport mechanism, while herbicides use ABC-type transporters (Klein *et al*., 1996; Frangne *et al*., 2002). Furthermore, transport studies revealed that the flavonoid glucoside saponarin, which occurs in barley but not in *Arabidopsis*, is taken up by an ABC-type transporter in isolated *Arabidopsis* vacuoles (Frangne *et al*., 2002). These results led to the hypothesis that plants contain two types of transporters for glucosylated compounds, antiporters for endogenous compounds and ABC transporters for foreign glucosylated compounds. However, this hypothesis is not true in all cases. ATP-dependent accumulation of glucosylated chlorsulphuron by vacuolar membrane vesicles, purified from red beet (*Beta vulgaris*) storage roots, is strongly inhibited by agents that either collapse or prevent the formation of a transmembrane $H^+$ gradient, but is completely insensitive to the phosphoryl transition state analogue vanadate, indicating that in this case, vacuolar uptake of a glucosylated xenobiotic is via a secondary activated transporter (Bartholomew *et al*., 2002).

There has been a long debate as to whether ABC transporters are involved in anthocyanin transport. Recently, Goodman *et al*. (2004) provided convincing genetic evidence that in maize this is the case. However, one should keep in mind that the modification of the anthocyanin basic structure is different in different plant species. In some plants, such as maize, anthocyanins are glycosylated and malonylated. The negative charge of the malonyl residue makes them potential substrates for MRP-type (multidrug resistance-related protein) ABC transporters. However, in other plants, such as *Arabidopsis*, anthocyanins are not malonylated. It will be interesting to investigate how the overall anthocyanin structures reflect the transport mechanism. A hint that different types of transporters could be involved in anthocyan transport comes from the finding that the *Arabidopsis* mutant *transparent testa 12* (*tt12*) exhibits strongly reduced proanthocyanidin deposition in the vacuole of seed endothelial cells. *TT12* codes for a MATE-type (multidrug and toxin efflux) transporter, which is a typical proton antiporter, suggesting that in this cell type a secondary activated transporter catalyses the uptake of proanthocyanidins (Debeaujon *et al*., 2001).

A further aspect which should be noted here is the increasing evidence that flavonoids are also present in the cytosol and that they can be used as indicators for altered auxin fluxes (Peer and Murphy, 2006). It is believed that flavonoids act as natural modulators of auxin transport, although the mechanism has to be elucidated. Interestingly, the *transparent testa 4* (*tt14*) mutant, which does not produce flavonoids because it lacks a chalcone isomerase gene, exhibits elevated auxin transport levels (Buer and Muday, 2004).

## Specialize to fulfil particular functions

A large number of signalling pathways are triggered by changes in cytosolic calcium concentrations. This means that cytosolic calcium concentrations must be kept low and constant, but change in response to abiotic and biotic stresses, such as light, touch, low and high temperature, oxidative stress, elicitors, or nodulation factors. In general,

increases in calcium concentration are generated by opening of calcium channels that allow calcium flows from both external and internal calcium stores into the cytosol (White and Broadley, 2003). To stop the signal, calcium must be subsequently removed from the cytosol.

The vacuole is the major internal calcium store. Some vacuolar calcium is present as calcium oxalate, some is chelated with organic acids, and some is present as free $Ca^{2+}$. The concentration of free $Ca^{2+}$ within the vacuole, which determines the $Ca^{2+}$ gradient between this organelle and the cytosol, is highly speculative. The vacuole is equipped with at least one vacuolar calcium pump (Geisler *et al*., 2000*b*), several $Ca^{2+}/H^+$ antiporters (Hirschi *et al*., 2004) that are responsible for calcium uptake, and a number of channels catalysing the efflux of calcium from the vacuole. Several excellent recent reviews are available describing current knowledge on this topic (White, 2000; Sanders *et al*., 2002; White and Broadley, 2003; Hirschi, 2004) and the different aspects of this important vacuolar function will only be briefly summarized, and some details of the CAX proteins, which, beside the vacuolar proton pumps, the aquaporins, and the $Na^+/H^+$ antiporters, are probably the best characterized tonoplast proteins, will be shown.

The vacuolar $Ca^{2+}$ pump is a P-type ATPase and mediates high-affinity $Ca^{2+}$ transport with an apparent $K_m$ in the low micromolar range (Geisler *et al*., 2000*a*, *b*). The $Ca^{2+}/H^+$ antiporters exhibit lower affinity ($K_m$ 10–15 μM), but higher capacities for calcium transport (Fig. 1). Several calcium channels mediate the efflux of calcium from the vacuole: an $IP_3$-dependent $Ca^{2+}$ channel, a cADPR-dependent $Ca^{2+}$ channel, a voltage-gated $Ca^{2+}$ channel, and possibly a TPC channel which is activated at elevated cytosolic $Ca^{2+}$ concentrations and corresponds to the well-described slow vacuolar (SV) channel (Peiter *et al*., 2005). However, it is still unclear whether *in vivo* the TCP1 channel can act as a $Ca^{2+}$ efflux channel. With the exception of the recently identified TCP1 channel (Peiter *et al*., 2005), the molecular nature of the vacuolar channels is still unknown.

The first plant $Ca^{2+}/H^+$ antiporter, named CAX1, was described by Hirschi *et al*. (1996). CAX proteins form a small family in the cation/$Ca^{2+}$ exchanger superfamily (Cai and Lytton, 2004). Seven members are present in the *Arabidopsis* genome (Cai and Lytton, 2004) and five in rice (Kamiya *et al*., 2005). Their function in modulating calcium accumulation in plants has been demonstrated by analysing mutants lacking CAX proteins or overexpressing *CAX* genes. Mutants lacking *CAX1* exhibit a 50% reduction in vacuolar $Ca^{2+}/H^+$ antiport activity, a 40% reduction in V-ATPase activity, and an up-regulation of *CAX3* and *CAX4*, indicating a complex regulation of vacuolar proton pump activity and a tendency to compensate for lack of CAX1 activity (Cheng *et al*., 2003). The *cax1* mutants exhibited altered development, with fewer lateral roots and a delayed transition from the vegetative to the flowering phase. Mutants lacking CAX3, which is also localized in the tonoplast, showed only a slight reduction in V-ATPase activity. However, the *cax1*/*cax3* double mutant exhibited a drastic phenotype with perturbed ion content (Cheng *et al*., 2005).

The CAX proteins are also involved in cellular responses to cold stress. CAX1 is induced by low temperatures, but not by abscisic acid (ABA). Mutants lacking CAX1 did not differ in their constitutive cold tolerance when compared with wild-type plants (Catala *et al*., 2003), but exhibited increased freezing tolerance after acclimation. It could be shown that this is probably due to increased transcript levels for several genes known to confer cold tolerance in *cax1* mutants during acclimation. Since CAX proteins also transport cations other than $Ca^{2+}$ (Hirschi *et al*., 2000), although these effects are likely to be due to impaired calcium signalling, it cannot be discounted that some effects are the result of a deregulated ion homeostasis.

Nevertheless, these results indicate the central role of the vacuole in calcium storage and demonstrate that the size of these stores must also be regulated to allow for optimal function. Several recent reports have elucidated important structural domains of CAX proteins (Kamiya and Maeshima, 2004; Pittman *et al*., 2005; Shigaki *et al*., 2005), such as the crucial role of the highly conserved His338 for transport activity. Interestingly, the mutation H338N reduced $Ca^{2+}$ transport activity to 25% of the wild-type activity but increased $Cd^{2+}$ and $Zn^{2+}$ transport activity. Regulatory elements such as the N-terminal autoinhibitory domain and the interaction of CAX1 with SOS2, a protein kinase involved in salt tolerance, have also been identified (Cheng *et al*., 2004*a*, *b*). These are the first steps to understanding how CAX1 interacts with the cellular calcium and heavy metal signalling and homeostasis networks.

## Be mighty because of your size

For efficient intracellular storage of solutes, the subcellular storage compartment has to occupy a substantial part of the total cell volume. Therefore, the central vacuoles, which usually occupy ~70–80% of the cell (Winter *et al*., 1993, 1994), are uniquely suited to store compounds such as organic acids, sugars, and inorganic ions. Since most of these solutes are involved in important processes such as CAM metabolism, cytosolic pH homeostasis, and the regulation of stomatal aperture, the impact of this large cell organelle on plant metabolism is beginning to be understood.

While cells of CAM plants tend to lose as little water as possible and not change size, guard cells are extremely dynamic and change their size in response to day/night rhythms and a large number of environmental stimuli. These changes induce stomatal opening and closing, and allow tight control of transpiration and $CO_2$ fixation. By

imaging guard cell vacuoles, Gao *et al.* (2005) analysed the dynamic changes of guard cell vacuoles during swelling and shrinking. They observed that the small vacuoles present in closed stomata readily fused and produced a complex network during stomatal opening, finally generating a large vacuole. Conversely, the large vacuole split into smaller vacuoles during stomatal closure and generated a complex internal membrane structure.

These rapid changes in size can only occur because water flow through biological membranes is catalysed by aquaporins. Aquaporins are the predominant plasma membrane and tonoplast proteins, and were originally discovered because they were the major tonoplast intrinsic proteins (Johnson and Ryan, 1990; Maeshima, 1992). Aquaporins constitute a large gene family. In *Arabidopsis*, 35 genes have been reported to code for aquaporins, while in maize and rice 33 genes constitute this family. Among them, 10 (*Arabidopsis*), 12 (maize), and 10 (rice) code for vacuolar-type (TIP) aquaporins. These TIPs are subdivided into five clades (Chaumont *et al.*, 2001; Luu and Maurel, 2005; Sakurai *et al.*, 2005). Expression levels of aquaporins are correlated with root hydraulic conductivity, indicating that regulation occurs mainly at the transcript level (Boursiac *et al.*, 2005). However, in addition to transcriptional regulation, post-transcriptional regulation of aquaporins has also been reported. It has also been shown that some TIPs are regulated through phosphorylation (Maurel *et al.*, 1995), as described for plasma membrane aquaporins (Törnroth-Horsefield *et al.*, 2006).

It remains to be determined whether pH changes in the cytosol affect the water permeability of vacuolar aquaporins, as was observed for plasma membrane aquaporins (Tournaire-Roux *et al.*, 2003). The highly conserved histidine residue, responsible for the pH dependence of plasma membrane aquaporins (PIPs), is not conserved in TIPs. Ma *et al.* (2004) reported that down-regulation of *AtTIP1;1*, the main tonoplast aquaporin of *Arabidopsis*, leads to plant death. Whether the compromised routing of carbohydrates observed in these plants is a consequence of reduced water circulation between the vacuole and the cytosol, or whether the phenotype observed is a result of impaired vesicle trafficking depending on *At*TIP1;1 as suggested by the authors, has yet to be determined. It is interesting to note that *Kalanchoë daigremontiana*, a typical CAM plant, contains only very low amounts of vacuolar aquaporins, as expected for a CAM plant whose water content does not fluctuate greatly (Maeshima *et al.*, 1994).

### *Function of vacuoles in CAM plants*

In most plant species, malate is involved in an extraordinarily wide array of functions. For example, in CAM plants, malate is synthesized at night and enters the vacuoles (as malic acid) temporarily as a storage form of carbon dioxide. Remarkably, in certain CAM species, an equivalent of 17% of the total cell dry mass crosses the tonoplast every single day (Holtum *et al.*, 2005). Thus, it is not surprising that a number of modifications of the central CAM vacuoles comprising both size and biochemical properties are required to allow such tremendous metabolic fluxes.

In this context, it is worth mentioning that vacuoles of CAM plants may occupy up to 95% of the mesophyll cell volume (Steudle *et al.*, 1980), possess densely packed and highly active V-type ATPase (Bremberger and Lüttge, 1988) to build up a steep proton gradient between the cytosol and the lumen, leading to vacuolar pH values of <2.9 (Franco *et al.*, 1990), and import malate down the resulting electrochemical gradient via highly active, but low abundance inward-rectifying malate channel proteins (Hafke *et al.*, 2003). Interestingly, the malate import capacity to vacuoles of *M. crystallinum* increases several times upon conversion from $C_3$ photosynthesis to CAM (Ratajczak *et al.*, 1994), indicating that the modifications above are part of complex inducible changes in certain CAM species.

Besides V-ATPases, the tonoplast of all plants analysed so far harbour a V-PPase that catalyses the transport of $H^+$ into the vacuole (Rea and Sanders, 1987, Rea *et al.*, 1992; Maeshima, 2000). Remarkably, in some CAM species, the activity of the tonoplast V-PPase is even higher than that of the V-ATPase (Marquardt and Lüttge, 1987), and from a thermodynamically point of view it is most likely that, under *in vivo* conditions, the V-PPase is not able to synthesize pyrophosphate, but permanently hydrolyses this compound (Drozdowicz and Rea, 2001).

Although it is difficult to clarify the relative contribution of the V-ATPase and the V-PPase to the energization of the vacuole, there is evidence that the relative activity of each enzyme depends upon the type of carbohydrate used for nocturnal malate synthesis. CAM plants can be divided into two groups according to the major carbohydrates used for malic acid synthesis: group 1 store starch during the day and use this compound for synthesis of malate in the night, whereas group 2 accumulate glucose and fructose (generated within the vacuole by invertase-catalysed hydrolysis of sucrose) during the day, and convert these compounds into precursors required for nocturnal malate synthesis (Holtum *et al.*, 2005). Experiments on tonoplast vesicles isolated from pineapple leaves (a typical monosaccharide-storing CAM plant) indicate that the tonoplast sucrose transporter (required for the subsequent generation of monosaccharides by vacuolar invertase activity) exhibits a $K_m$ value of ~50 mM, was *trans*-stimulated by internal sucrose, and is specific for di-, but not for monosaccharides (McRae *et al.*, 2002). It may be noted that despite the large number of publications on vacuolar sucrose and hexose transport (for a review, see Martinoia *et al.*, 2000), so far no vacuolar sugar transporter has been characterized at the molecular level.

Interestingly, starch-degrading CAM species exhibit a tonoplast V-PPase/V-ATPase activity ratio which is ~3–4 times higher than that in monosaccharide-degrading CAM species (Chen and Nose, 2004). Starch-degrading CAM plants exhibit a high tonoplast V-PPase activity because they are able, in contrast to species using monosaccharides, to generate malate by phosphorylase activity (Holtum *et al*., 2005). Through this enzyme, they save energy, leading to high nocturnal ATP levels and promoting a futile cycle-between ATP-phosphofructokinase and phosphofructo-phosphotransferase (PFP), leading to a release of cytosolic PPi. Therefore, starch-degrading species require a highly active V-PPase. Unfortunately, due to the lack of a suitable transformation protocol, reverse genetic approaches have not been successful on any CAM species to date. However, it will be interesting to study the effect of altered V-ATPase or V-PPase activity on the nocturnal and photosynthetic performance of such mutants in the future.

### *Function of vacuoles in pH homeostasis*

Cytosolic pH homeostasis is important because most enzymes in this compartment exhibit their highest activity at ~pH 7. The early observation that the cytosolic pH is very stable, even under artificial conditions of extremely low external pH values (Raven and Smith, 1978), indicated that the proton concentration in this cellular space is strictly regulated.

Both theoretical considerations and experimental data support the assumption that malate plays a fundamental role in the regulation of cytosolic pH. This is because during the synthesis of malate via PEP carboxylase, protons are released into the cytosol, whereas during malate degradation via malic enzyme, $OH^-$ ions are released (Smith and Raven, 1979). As acidification activates malate-degrading malic enzymes, but inhibits PEP carboxylase (Davies, 1986) and vice versa, an altered ratio between malate synthesis and degradation is crucially important for cytosolic pH homeostasis (Kurkdjian and Guern, 1989).

Having now in mind that most of the cellular malate in plants is located in the vacuole (Gerhardt and Heldt, 1984), one might speculate that the central vacuole is involved in pH homeostasis. In fact, this assumption is supported by the following observations: first, as predicted above, acidification of mesophyll cells leads to a stimulation of malate degradation in *Arabidopsis* leaves (Hurth *et al*., 2005), or in storage cells from wheat aleurone tissue (Martinez-Camacho *et al*., 2004). In contrast, alkalinization promotes the synthesis of malate and citrate, as a product of malate synthesis (Gout *et al*., 1992), and subsequently leads to an accumulation of these carboxylates in the vacuole (Gout *et al*., 1993). Secondly, acidification of the plant cytosol stimulates the expression of the gene coding for the tonoplast malate transporter *At*tDT (Hurth *et al*., 2005). Thirdly, knock-out plants lacking a functional vacuolar malate transporter *At*tDT exhibit impaired ability to maintain metabolic processes, such as photosynthesis in mesophyll cells, in response to acidification of the cytosol (Hurth *et al*., 2005).

The observations that the tonoplast malate carrier protein *At*tDT is able to import malate into isolated *Arabidopsis* vacuoles (Emmerlich *et al*., 2003), and that the corresponding gene is markedly induced during acidification of the cytosol (Hurth *et al*., 2005)—representing conditions of increased malate consumption (Smith and Raven, 1979; Gout *et al*., 1992)—indicates that this carrier is less inward rectifying than the strongly inward rectifying malate channel protein (Hafke *et al*., 2003). However, because the molecular nature of the tonoplast malate channel protein is still elusive, it remains to be determined to what extent the latter protein might also be involved in regulating cytosolic pH. Thus, according to these considerations, it is predicted that the vacuolar malate transporter *At*tDT is critical for controlling the cytosolic pH homeostasis (Fig. 2).

### *Function of vacuoles in guard cells*

It is commonly known that transient changes in the concentrations of potassium ions and malate in guard cells are of primary importance for changing stomatal aperture (Fischer, 1968; Allaway, 1973). Changes in guard cell potassium concentrations are mediated by $K^+$-specific channel proteins, located in the plasma membrane, that allow the import and export of this solute (Roelfsema and

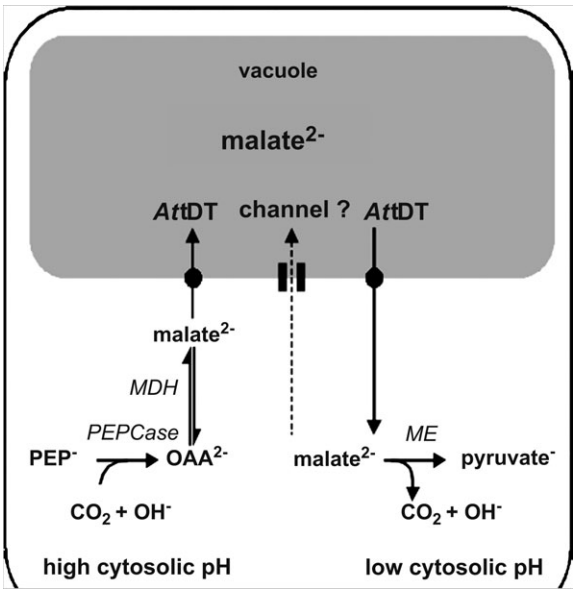


**Fig. 2.** The role of the vacuolar malate transporter for cytosolic pH homeostasis. At high cytosolic pH, phosphoenolpyruvate is carboxylated and $OH^-$ is used to produce oxaloacetate which is readily reduced to malate by cytosolic malate dehydrogenase. Malate is transported into the vacuole as $malate^{2-}$. At low cytosolic pH, malate is released from the vacuole, decarboxylated by malic enzyme, and $OH^-$ is released.

Hedrich, 2005). In contrast, malate synthesis and degradation are a consequence of enzymatic reactions in the cytosol.

Inhibition of PEP carboxylase by application of a specific enzyme inhibitor demonstrates that malate synthesis is a prerequisite for a controlled opening of stomata (Asai *et al*., 2000). Because PEP carboxylase is present solely in the cytosol (Holtum and Winter, 1982; Holtum *et al*., 1986), and since the guard cell enzyme is similar to PEP carboxylases from other plant cells (Westhoff and Gowik, 2004) that are inhibited by rising malate concentrations (Tarczynski and Outlaw, 1993), an efficient removal of malate from the cytosol by uptake into the vacuole is a prerequisite to build up a sufficiently high osmotic pressure required for stomatal opening (for a recent comprehensive review on stomatal physiology, see Roelfsema and Hedrich, 2005).

Although the presence of an anion-specific channel protein in guard cell tonoplasts with similarities to the anion inward rectifier in mesophyll cells (Hafke *et al*., 2003) has been demonstrated (Pei *et al*., 1996), it is still uncertain to what extent this protein controls malate movement across this membrane. In fact, as an alternative or in addition to the guard cell tonoplast anion channel (Pei *et al*., 1996), the recently identified vacuolar malate carrier *At*tDT (Emmerlich *et al*., 2003) might contribute to the regulation of stomatal aperture. This assumption is consistent with the observation that expression of the *AttDT* gene (as reported by promoter–reporter gene analysis) is very high in *Arabidopsis* guard cells when compared with the surrounding mesophyll cells (E Neuhaus, MA Hurth, M Klein and E Martinoia, unpublished data). Thus, it will be challenging to analyse guard cell physiology and regulation of stomata aperture in *AttDT* knock-out mutants in detail.

Cytosolic $Ca^{2+}$ is known to be a universal second messenger in all eukaryotic cells and is central to the regulation of stomatal aperture (Poole, 1993). Interestingly, guard cells exhibit an ABA-induced increase in cytosolic $Ca^{2+}$ concentrations (Gilroy *et al*., 1991). Because ABA application to guard cells also causes a rapid decrease of turgor pressure, and subsequent closure of stomata (Leckie *et al*., 1998), and since rising $Ca^{2+}$ levels activate anion channels in the guard cell plasma membrane (Hedrich *et al*., 1990), the origin of the ABA-induced increase in cytosolic $Ca^{2+}$ has been the subject of much research. In fact, tonoplast $Ca^{2+}$ channel activity is indirectly stimulated by increasing ABA concentrations (Allan *et al*., 1994; Leckie *et al*., 1998), but none of the compounds involved in the transmission of the ABA signal to the channel proteins has been identified at the molecular level (Roelfsema and Hedrich, 2005).

The observation that mutants lacking the two-pore channel protein TPC1 (Peiter *et al*., 2006), which corresponds to the well-described SV channel, is impaired in $Ca^{2+}$-dependent, but not ABA-dependent stomatal closure is a milestone in understanding the impact of vacuoles on stomata physiology. However, the role of TPC1 in vacuolar $Ca^{2+}$ release is still unsolved (Ward and Schroeder, 1994; Bewell *et al*., 1999; Pottosin *et al*., 2001; Ivashikina and Hedrich, 2005) and, because the channel is relatively non-selective among mono- and divalent cations, the phenotype observed might derive either from altered potassium fluxes and distribution or from TPC1-dependent $Ca^{2+}$ signalling. Despite the vast amount of information on this channel, its role is controversial and will depend on a wide variety of factors known to modulate its activity, such as internal free calcium concentrations, *in vivo* threshold activation potential, redox potential, pH, and phosphorylation (White, 2000). Interestingly, with respect to the guard cell phenotype, mutants lacking TCP1 mirror the *det3* mutant which lacks vacuolar $H^+$-ATPase activity (Allen *et al*., 2000) and also exhibits impaired responses to external $Ca^{2+}$ but not to ABA.

For stomatal movement, potassium ions are most important (Fischer, 1968). During changing stomatal aperture, $K^+$ ions cross the guard cell plasma membrane, probably via an NHX ($Na^+$,$K^+$/$H^+$ antiporter) and various potassium-specific channels proteins, classifiable as either outward rectifiers, inward rectifiers, or voltage-dependent potassium channel proteins (Roelfsema and Hedrich, 2005). In guard cells, SV, fast vacuolar (FV), and potassium-specific vacuolar (VK) $K^+$ channels have been demonstrated. Furthermore, TPK1 (KCO1), a member of the two-pore channel family, localized in the vacuolar membrane (Czempinski *et al*., 2002), has recently been characterized using heterologous expression in yeast (Bihler *et al*., 2005). The properties of this channel resemble those described for the VK channel from *Vicia faba* ($Ca^{2+}$-activated, $K^+$-selective and voltage-independent). In detailed experiments Allen and Sanders (1996) demonstrated that individual *V. faba* guard cell vacuoles contained FV, VK, and SV channels, and that each type of channel was differentially regulated by cytosolic $Ca^{2+}$. FV-type channels were active at low $Ca^{2+}$ levels, whereas SV channels were activated by higher cytosolic $Ca^{2+}$ concentrations (Allen and Sanders, 1996). This response of tonoplast $K^+$ channels to altering cytosolic $Ca^{2+}$ concentrations, which may also be influenced by the activity of other vacuolar channel proteins (Peiter *et al*., 2006), further underlines the high impact of vacuoles on plant metabolism.

## Where does this train go?

It has been argued that research on vacuolar transport is also research on cytosolic metabolism. The availability of deletion mutants for most *Arabidopsis* genes allows researchers not only to verify the transport activity of a tonoplast transporter but also to analyse the effect of impaired vacuolar transport on cellular metabolism. In this

respect, we are just at the beginning of a new area of research.

It has been demonstrated that deletion mutants for the $Na^+/H^+$ or $Ca^{2+}/H^+$ antiporters have drastic effects on plant development, and that deletion mutants for the vacuolar sulphate transporter exhibit altered sulphate content. The analysis of the vacuolar malate transporter revealed many aspects which were not expected (Hurth *et al*., 2005). The absence of this transporter caused decreased vacuolar malate and fumarate contents, resulted in an increased vacuolar citrate content, and changed the respiratory quotient from ~1.1 to 1.3, indicating that while in wild-type plants the respired substrates are derived mainly from carbohydrates, decreasing the vacuolar storage capacity switched to the respiration of mainly carboxylic acids.

However, these are all just first hints that show how important a tight regulation of the vacuolar stores is. A more in-depth understanding is needed about what is changing, and in which compartment metabolite changes occur. A 'metabolomic' approach will help to elucidate many aspects of impaired homeostasis, but, in most cases, only a compartment-specific metabolic analysis will really give the answers required to understand the complex interactions between vacuolar transport and plant metabolism. Looking at the data published in the last years and realizing how tightly vacuolar transport must be regulated, it is also astonishing that so little is known about the regulation of vacuolar transporter activity. An aspect in regulation that should be taken into account is the interaction between different proteins regulating transport activity, as shown for the interaction between the immunophilin-like TWD1 protein and the vacuolar ABC transporter AtMRP1 (Geisler *et al*., 2004). Combining 'metabolomic' analysis with transcript analysis should reveal at which level metabolic pathways are regulated and also provide insights about control of vacuolar transport processes.

An additional point is that so far, only a small portion of vacuolar membrane transporters have been identified. A method to obtain more insights into the vacuolar proteome is to isolate highly enriched vacuolar preparations and subject them to a 'proteomic' analysis. Such an approach has been undertaken and published for *Arabidopsis*. (Carter *et al*., 2004; Sazuka *et al*., 2004; Shimaoka *et al*., 2004; Szponarski *et al*., 2004) and very recently for barley vacuoles (Endler *et al*., 2006).

Each group used different purification and separation methods. Interestingly, the overlapping of the identified proteins is very low. Between 34 and 402 different proteins, including soluble and membrane proteins, were detected. From these, 23–112 were reported to be vacuolar membrane proteins. This discrepancy can be attributed to the different purities of membranes, and different techniques used in each approach. In addition to vacuolar membrane proteins, non-vacuolar proteins from other organelles such as mitochondria, plasma membrane, and chloroplasts were also detected. Therefore, for all newly identified putative vacuolar proteins, further experimental proof for vacuolar localization is required in order to confirm their vacuolar location. However, so far, this aspect has been addressed only for barley vacuoles, where it could be shown that HvSut2 and, surprisingly, AtSUT4 are vacuolar sucrose transporters.

Meanwhile, it has been demonstrated for many transporters that members of the same family can be located on the vacuolar membrane as well as on the plasma membrane. This is, for example, the case for aquaporins (TIP and PIP), $Ca^{2+}$-ATPase, MTPs (*At*MTP1 is in the tonoplast and *At*MTP4 resides in the plasma membrane), ABC transporters, and NHXs. The presence of very similar transporters on two membranes indicates that plants have the choice to excrete compounds which are not required or which inhibit metabolism into the apoplast (excretion), or to transport them into the vacuole (internal excretion; Martinoia *et al*., 1993). Excretion to the apoplast as well as internal excretion provides the conditions for optimal cell metabolism. However, the result is different. Excretion of water-soluble compounds into the apoplast will result in the excreted compound moving towards the stomata, with the consequence that other cells will become exposed to this compound.

Such an excretion may be important for compounds where a cell sensed that the internal concentration is approaching toxic concentrations. The observation that at high cadmium concentrations the proportion of cadmium present in epidermal cells increases (H Winter and E Martinoia, unpublished data) may indicate that metabolically active mesophyll cells excrete this toxic heavy metal which is transferred to metabolically less active cells. Furthermore, if a cell excretes more hydrophobic compounds, which can react with the cell walls, these compounds will be bound and no longer available for a plant cell.

In contrast, internal excretion into the vacuole will allow a cell to reuse compounds when required by the metabolism. Furthermore, potentially toxic compounds that defend the plant against pathogens and herbivores are only efficient if they are in a soluble form and readily available.

These considerations show that both plasma membrane transporters and vacuolar transporters play an important role in maintaining cytosolic homeostasis, but nothing is known about how these reciprocal transport processes are regulated. Furthermore, and as mentioned in the Introduction, different vacuoles within one organ may play different roles and actually the present view on vacuoles is still oversimplified.

Furthermore, it should also be kept in mind that different plants possess contrasting metabolic pathways. Very little is known about the differences in the metabolism of palisade and spongy parenchyma, but it is tempting to speculate that these two cell types are at least partially

specialized and that the corresponding vacuoles have partially different functions. This aspect is even more pronounced if vacuoles in *Arabidopsis* are compared with corresponding organelles in CAM plants. To elucidate the role, therefore, efforts should also be made to elucidate specific roles of vacuoles in plants exhibiting specific metabolic pathways. Such an approach would teach us about the multifunctionality and flexibility of plant vacuoles.

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