

Mini Review

Transport Processes of Solutes across the Vacuolar Membrane of Higher Plants

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The central vacuole is the largest compartment of a mature plant cell and may occupy more than 80% of the total cell volume. However, recent results indicate that beside the large central vacuole, several small vacuoles may exist in a plant cell. These vacuoles often belong to different classes and can be distinguished either by their contents in soluble proteins or by different types of a major vacuolar membrane protein, the aquaporins. Two vacuolar proton pumps, an ATPase and a PPase energize vacuolar uptake of most solutes. The electrochemical gradient generated by these pumps can be utilized to accumulate cations by a proton antiport mechanism or anions due to the membrane potential difference. Uptake can be catalyzed by channels or by transporters. Growing evidence shows that for most ions more than one transporter/channel exist at the vacuolar membrane. Furthermore, plant secondary products may be accumulated by proton antiport mechanisms. The transport of some solutes such as sucrose is energized in some plants but occurs by facilitated diffusion in others. A new class of transporters has been discovered recently: the ABC type transporters are directly energized by MgATP and do not depend on the electrochemical force. Their substrates are organic anions formed by conjugation, e.g. to glutathione. In this review we discuss the different transport processes occurring at the vacuolar membrane and focus on some new results obtained in this field.

Key words: ABC transporters — Channels — Compartmentation — Transport — Vacuole.

Introduction

Until some years ago the vacuole was described as the largest compartment of a mature plant cell which may occupy more than 80% of the total cell volume. Furthermore, protein bodies were described as specialized vacuoles occurring in seeds. Recently, it was shown that, despite the similarities of these compartments, they can be differentiated and that cells do not contain just one vacuole and prevacuoles generating the vacuole (Paris et al. 1996, Jauh et al. 1999). Vacuoles can be differentiated by their soluble proteins and by the class of aquaporins localized in their respective membranes. It is generally accepted that α -TIPs are localized on storage vacuoles and γ -TIPs on the membrane of lytic vacuoles (Jauh et al. 1999). δ -TIPs have been reported to be associated with both types of vacuoles; however, it appears that a third vacuole type exists, in which δ -TIPs are exclusively localized (Jauh et al. 1999). Since a cell probably contains several provacuoles together with different types of vacuoles, it may therefore be more correct to use the term 'vacuome', to indicate the sum of all vacuoles within a cell. Nevertheless, the general description of the large, central vacuoles is still true: they contain mainly inorganic salts and water, enabling the plant to reach a large size and surface area using a minimum of energy for the synthesis of organic metabolites (Matile 1987, Martinoia 1992). The vacuole also serves as an internal reservoir of metabolites and nutrients and takes part in cytosolic ion homeostasis (Boller and Wiemken 1986, Matile 1987, Martinoia 1992, Leigh 1997). Vacuolar constituents vary between and within plant species, depending on the environmental conditions, suggesting that transport of metabolites and nutrients across the tonoplast is strictly controlled to permit an optimal functioning of the cytoplasm. However, because several types of vacuoles exist within one tissue and even within one cell, it is possible that contradictory results may be obtained if vacuolar transport processes are determined using purified tonoplast vesicles or intact vacuoles. This may be the reason why a vacuolar Ca-ATPase (Chanson 1993) was described for tonoplast vesicles, but no such activity could be detected in isolated barley mesophyll vacuoles (Gaillard and Martinoia unpublished). Furthermore, it must be emphasized that due to the different surface/volume ratios of small vacuoles and the central vacuole the energization status of the two vacuolar types may be different and that some compounds may accumulate to a far higher degree in small vacuoles. Fusion of small vacuoles with the large central vacuole may be an accumulating mechanism not considered enough up to now.

The scope of this review is to give a short overview on transport processes across the vacuolar membrane for non-specialists and to stress some recent results. For further reading, several reviews on specialized topics of vacuolar solute transport have been published recently (Martinoia 1992, Wink 1997, Leigh 1997, Allen and Sanders 1997, Blumwald and Gelli 1997, Martinoia and Ratajczak 1997, Rea et al. 1998, Rea

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1999, Martinoia et al. 2000).

Proton pumps

Proton pumps play a central role in the function of the tonoplast by generating a transmembrane H^+ electrochemical gradient which can be utilized to drive the transport of solutes (Fig. 1). The tonoplast contains different proton pumps, an ATPase and a PPase. V-ATPases (vacuolar-type) are present on different membranes of eukaryotic cells (Nelson and Taiz 1989). The tonoplast H^+ ATPase is specifically inhibited by bafilomycin A1 and concanamycin A (Dröse et al. 1993) in the submicromolar range and, like FoF1 ATPases, by NO_3^- . The presence of Cl^- has been shown to stimulate V-ATPase activity. In contrast, vanadate, which inhibits the plasma membrane H^+ -ATPase, usually does not affect the vacuolar proton pump. However, in a very few cases, vanadate inhibition has been observed (for a review see Sze et al. 1999). Azide and oligomycin, which inhibit the F-ATPases (present on the plasma membrane of eubacteria and on the inner membranes of mitochondria and chloroplasts), have no effect. V-ATPases are constituted of 13 subunits and show many similarities to F-ATPases (Nelson and Taiz 1989, Sze et al. 1999). The tonoplast PPase, an enzyme strongly stimulated by K^+ present at the cytosolic face of the tonoplast (Rea and Sanders 1987, Maeshima and Yoshida 1989, Rea and Poole 1993, Zhen et al. 1997, Maeshima 2000), is an integral entity of the tonoplast. It consists of one 80 kDa subunit and no homologue has been described in fungi or animals. The vacuolar PPase is inhibited by 1,1-diphosphonates.

Aquaporins

For a long time, it was assumed that water crosses biological membranes by diffusion through the lipid bilayer. However, it was noticed that the water permeability of biological membranes was greater than that through a lipid bilayer and that water fluxes through biological membranes could be partially inhibited by mercury, suggesting that either water can cross the membrane through specific water pores or through transport proteins together with solutes. Indeed, it was shown that the transfer of some metabolites, e.g. glucose, is accompanied by the transport of water. However, the real causes for the discrepancy observed between lipid bilayers and biological membranes are the aquaporins. The fact that some small, very hydrophobic proteins, so-called MIPs (major intrinsic membrane proteins) are abundantly present in membranes was already observed in 1984 (Gorin et al. 1984). However, the discovery that many of the MIPs form water channels was made much later (Preston et al. 1993). In plants, membrane proteins homologous to MIPs were first identified as major proteins of the peribacteroid membrane (Fortin et al. 1987). A few years later, Johnson et al. (1989) described that the most abundant protein in the protein body membrane is also a member of the MIPs and it was called α -TIP (tonoplast intrinsic protein). This was also true for the major membrane protein of the central

vacuole of radish, the γ -TIP (Maeshima 1992). Both TIPs have been shown to act as water channels. α -TIP is associated with the storage vacuole while the γ -TIP is localized on the lytic vacuole. Interestingly, α -TIP has to be phosphorylated in order to exhibit water channel activity (Maurel et al. 1995). α -TIP is expressed mainly in storage tissues (Johnson et al. 1989), whereas γ -TIP is expressed in young developing organs like hypocotyls (Ludevid et al. 1992). A strongly increased expression of γ -TIPs has also been demonstrated in motile pulvini of *Mimosa pudica* compared to young, non-active pulvini (Fleurat-Lessard et al. 1997), suggesting that increased water permeability is required for cell motility. During the Arabidopsis sequencing project, it became evident that a large number of genes belong to the MIP family. Future work will show whether other members of this family are associated with the vacuolar membrane. For further reading consult Chrispeels et al. 1997, Maurel 1997 and Hara-Nishimura and Maeshima 2000.

Transport of products of the primary metabolism

During the light period, many metabolites are accumulated in the leaves which, during the dark period, are either transported to sink tissues or degraded in the Krebs cycle. Experiments using protoplasts and rapid vacuole isolation have shown that products of the primary metabolism are rapidly transferred into the central vacuole (Kaiser et al. 1982, Boller and Alibert 1983). In a very careful compartmentation study, Gerhardt et al. (1987) showed that the diurnal changes of the malate content in spinach could be attributed to changes in the vacuolar malate content; negligible changes were observed in the cytosol and stroma. These results indicate that the vacuole plays an important role as an intermediate storage compartment for products of primary metabolism in order to maintain the cytosolic homeostasis necessary for metabolism.

Carbohydrates—In leaves, carbohydrates accumulate during the day, when the loading capacity of phloem is limiting, and are exported during the night. In storage tissues, they are accumulated during a vegetation period and utilized as a source of energy for growth in the subsequent period. For carbohydrate metabolism, the role of a leaf vacuole is therefore different from a vacuole of a storage tissue. Leaf vacuoles must be equipped with a transport system enabling a rapid accumulation and export of sucrose as a function of the physiological status. Experiments using isolated leaf vacuoles showed that sucrose uptake occurs by facilitated diffusion (Kaiser and Heber 1984, Martinoia et al. 1987a), a transport mechanism which allows rapid equilibration between the cytosol and vacuole. The permease has a low affinity for sucrose (K_m 20–30 mM, a concentration which is easily attained in some plants during the daytime) and is not inhibited by hexoses. Facilitated diffusion of sucrose rather than active transport of sucrose has been also observed for vacuoles isolated from sugar cane cell cultures, which accumulate sucrose at concentrations comparable to those in the stalk tissue (Preissner and Komor 1991) and tomato fruit vacuoles (Milner et al. 1995).

In contrast to sucrose uptake in leaf vacuoles, its uptake in vacuoles of root storage tissue may be energized. For red beet, sucrose transport was found to be stimulated by MgATP and to occur via a sucrose/H⁺ antiport (Doll et al. 1979, Briskin et al. 1985). The affinity of this active carrier was similar to the passive one of barley leaf vacuoles (approximately 20 mM). Larger carbohydrates, as shown for stachyose, a member of the raffinose family which is present in large quantities in *Stachys sieboldi*, may also be accumulated in the vacuole by proton antiport mechanisms (Keller 1992).

An alternative way to accumulate soluble carbohydrates within the vacuole without energizing the uptake of sugars involves the synthesis of fructans, which are water soluble carbohydrates with polymerization grades of 3–50 fructose molecules, found at high concentrations in different tissues of cereals and many other plant species. Their synthesis occurs within the vacuole (Wagner et al. 1983, Frehner et al. 1984). In a first step, the enzyme sucrose-sucrose-1-fructosyltransferase (Pollok and Cairns 1991) transfers a fructan moiety to a sucrose, yielding a trisaccharide which is trapped in the vacuole (Heck and Martinoia, unpublished) and a glucose which can be exported from the vacuole by facilitated diffusion (see below, Martinoia et al. 1987a) to be remetabolized in the cytosol.

As for sucrose, both facilitated diffusion and active transport has been described for vacuolar uptake of glucose. For barley (Martinoia et al. 1987a), celery (Daie and Wilusz 1987) and pear fruit vacuoles (Shiratake et al. 1997), facilitated diffusion has been demonstrated, while an active proton antiport mechanism has been postulated for pea vacuoles (Guy et al. 1979), sugar cane (Thom and Komor 1984), and maize coleoptile tonoplast vesicles (Rausch et al. 1987). A large number of plasma membrane sucrose and glucose transporters have been identified during the last few years (Kühn et al. 1999). In contrast, no vacuolar sucrose or glucose transporter has been unequivocally identified to date. Chiou and Bush (1996) reported the cloning and vacuolar localization of a putative sugar transporter from sugar beet. However, the authors did not demonstrate transport activity, and more detailed localization studies are needed to prove that the gene product indeed codes for a vacuolar sugar transporter.

Furthermore, it must be mentioned that accumulation of hexoses within the vacuole, an observation which has often been reported (e.g. Preissner and Komor 1991), is not a proof for energized hexose uptake, because acid invertases are localized in the vacuolar sap (Boller and Wiemken 1986).

Sugar alcohols are found in many plants and may accumulate within the vacuoles. In immature apple fruit tissue, transport of sorbitol across the tonoplast appears to be ATP-dependent (Yamaki 1987). It is not yet known whether this permease is specific for sorbitol. A different situation is found in celery petioles, in which mannitol appears to be equally distributed between the extravacuolar space and the vacuole (Keller and Matile 1989). Indeed, transport experiments suggest that mannitol crosses the tonoplast by facilitated diffusion (Greutert et

al. 1998).

Amino acids—In contrast to fungi, our knowledge about amino acid transport across the tonoplast of higher plants is limited. The most prominent amino acid transport system, observed first in barley (Dietz et al. 1990, Martinoia et al. 1991, Görlach and Willms-Hoff 1992) and subsequently in other plants (Thume and Dietz 1991), is a carrier or channel which is modulated by free ATP (but not by MgATP) which induces inward as well as outward (Dietz et al. 1989) fluxes of all amino acids tested. ATP hydrolysis is not required because the non-hydrolysable ATP-analogue AMPPNP is also able to induce amino acid fluxes through the vacuolar membrane. However, most of the ATP present in the cell is complexed to magnesium and free ATP concentrations necessary to induce maximal amino acid fluxes are much higher (4–5 mM) than those expected to occur in the cytosol (50 μM). Taking advantage of the fact that paraquat, a herbicide not metabolized within the cell, is transported into isolated vacuoles like amino acids, only in presence of free ATP, Mornet et al. (1997) analyzed the compartmentation of paraquat taken up into barley protoplasts. After an 1 h incubation, 20–30% of the paraquat was present within the vacuole, indicating that the ATP-dependent channel is partially open in vivo. Stimulation of amino acid transport by ATP is inhibited by hydrophobic amino acids such as L-phenylalanine and L-leucine but not by D-phenylalanine or hydrophobic amines or carboxylates such as phenylethylamine and phenylpropanecarboxylic acid. Thume and Dietz (1991) succeeded in the reconstitution and partial purification of the vacuolar amino acid transporter. The fact that one transport system recognizes all or at least most amino acids would suggest that the amino acid content would reflect more or less (differences may be due to different uptake velocities for the different amino acids) the content of amino acids present in the cytosol. Indeed, for *Chara* cells, it has been shown that the ratio of the different amino acids between the cytosol and vacuole is quite constant, indicating that, in this organism, there is no preferential uptake of a specific amino acid (Mimura et al. 1990). In higher plants the situation may be slightly different since in addition to the described uptake system an energized uptake of phenylalanine (Homeyer et al. 1989) as well as a ATP-independent uptake of positively charged amino acid has been demonstrated (Martinoia et al. 1991).

It must be mentioned that compartmentation experiments (Martinoia and Ratajczak 1997) indicate that the amino acid concentrations are usually lower in the vacuole than in the cytosol. The release of amino acids stored in the vacuole must therefore be energy-dependent. Indeed, in the giant algae *Chara australis*, export of alanine and glutamine from the vacuole is accompanied by a rise in vacuolar pH, indicating the existence of a proton/amino acid symport (Amino and Tazawa 1989) for the extrusion of amino acids. However, for higher plants, no such an activity has yet been described.

Organic acids—Among the different organic anions often present at high concentrations in plants, malate transport across

the vacuolar membrane has been studied most intensively. This is due to the central role of malate in plant metabolism (Martinoia and Rentsch 1994) and the fact that vacuoles are important temporary stores for this organic acid. Malate transport has been investigated using flux experiments as well as the patch clamp technique. The results obtained with the two techniques show that malate uptake is driven by the electrical component of the electrochemical potential generated by the proton pumps. Uptake of malic acid into barley mesophyll vacuoles was not specific for the natural enantiomer L-malic acid. D-malic acid, citric acid, and other di- and tricarboxylic acids behaved as competitive inhibitors (Martinoia et al. 1985, White and Smith 1989, Martinoia and Ratajczak 1997). Calculation of the K_m values at different external pHs suggests that the malate dianion is transported across the tonoplast (Rentsch and Martinoia 1991).

In addition to experiments using radiolabeled malate, the patch clamp method was extensively used to determine fluxes of malate across the tonoplast. Two channels exhibiting malate permeability have been demonstrated (Iwasaki et al. 1992, Pantoja et al. 1992a, Cerana et al. 1995, Cheffings et al. 1997). One could be responsible for the efflux of malate. The other is very strongly inward rectifying, corresponding to malate uptake in the vacuolar lumen, and exhibits properties similar to those of the uptake system described using radiolabeled malate. Indeed, this channel also mediates uptake of succinate, fumarate, and oxaloacetate. The malate channel is not affected by cytosolic Ca^{2+} or ATP. Re-entry of malate to the cytosol may be blocked by vacuolar chloride, which has been reported to inhibit the malate channel (Plant et al. 1994). These observations raise the question of whether the di/tricarboxylate transporter identified using tracer flux analysis and the patch clamp technique are identical. An argument against this hypothesis is the low K_m observed in experiments using radiolabeled malate. However, it cannot be excluded that the higher membrane potential differences applied in order to observe currents with the patch clamp technique may alter the affinity of the transporter/channel. A very interesting observation was made by Pei et al. (1996): they identified a vacuolar chloride channel (VCL) which is strongly activated by phosphorylation and also exhibits malate conductance (see the section on chloride).

Martinoia et al. (1991) and Steiger et al. (1997) succeeded in the functional reconstitution of a highly purified malate carrier. These data indicate that the malate transporter is a 32 kDa protein. However, up to now, this transporter has not been cloned. A 21 kDa polypeptide identified by affinity labeling using *N'*-(2-hydroxy-5-azido)-diazot-N-4-[^3H]3,5-benzenedicarboxylic as a citrate analogue and purified from the vacuole-like luteloids of *Hevea brasiliensis* was found to be a hydrophilic, partially membrane associated protein of lutoids (Rentsch et al. 1995).

In most systems investigated, it has been suggested that citrate crosses the tonoplast using the same transporter as

malate (Oleski et al. 1987, Rentsch and Martinoia 1991). From kinetic data, it is assumed that citrate $^{3-}$ is the transported form. A different mechanism is probably involved in citrate uptake in lutoid membrane vesicles of *H. brasiliensis* (Marin et al. 1982, Rentsch et al. 1995). The MgATP-dependent accumulation was suggested to occur by a $\text{H}_2\text{citrate}^-/\text{H}^+$ antiport by an experiment using lutoid membrane vesicles and a partially purified citrate transporter reconstituted in proteoliposome.

Assuming that most of the di- and tricarboxylic acids cross the tonoplast by the same carrier, the question still remains as to whether a separate transport system may exist for monocarboxylic acids like ascorbate or shikimate, which may be present in high amounts in plant cells. In the case of ascorbate it has been shown that it crosses the membrane of isolated barley vacuoles very slowly by diffusion (Rautenkrantz et al. 1994). However, the ascorbate concentration in barley vacuoles is very low, and the situation may be different in plants accumulating high concentrations of ascorbate in the vacuole, such as horseradish or other members of the crucifera family.

Uptake of inorganic anions

Compartmental analysis, together with membrane potential measurements, have indicated active transport of Cl^- (Pierce and Higinbotham 1970, MacRobbie 1970). With the discovery of the electrogenic pumps at the tonoplast this phenomenon was readily explained: the H^+ pumps generate a positive potential inside the vacuole, thus providing the driving force for anion movements (Fig. 1). Anion-dependent dissipation of a proton-pump generated by anions revealed that NO_3^- permeated more rapidly than Cl^- ; SO_4^{2-} and HPO_4^{2-} crossed the tonoplast very slowly (Churchill and Sze 1984).

Chloride—Kästner and Sze (1987) studied the potential-dependent anion movement in tonoplast vesicles from oat roots using the fluorescent probe oxanol V. The relative permeability of the anions was $\text{SCN}^- > \text{NO}_3^- = \text{Cl}^- > \text{SO}_4^{2-} = \text{HPO}_4^{2-}$. Dissipation of the membrane potential by chloride and nitrate was saturable with K_m of 2.3 and 5 mM respectively. Similar results have been published by Pope and Leigh (1987), using the same material. Unfortunately, this approach does not allow one to determine whether these anions cross the tonoplast by the same or by different carriers. In intact barley mesophyll vacuoles, ATP-dependent Cl^- uptake was detected using $^{36}\text{Cl}^-$ (Martinoia et al. 1986). Concentration-dependent uptake exhibited a saturable component with a K_m of 2–3 mM as well as a linear component. Chloride uptake was competitively inhibited by nitrate (K_i 5 mM) (Martinoia et al. 1987b). Comparable results were obtained with an alternative method by Pope and Leigh (1988), Pope and Leigh (1990) and Pope et al. (1990) utilizing the Cl^- sensitive fluorescent dye 6-methoxy-1-(3-sulfonatopropyl)quinolinium. These authors furthermore showed that the voltage-dependent uptake of chloride exhibits a sigmoidal response at Cl^- concentrations up to 30 mM.

In comparison to cation channels, much less is known about vacuolar Cl^- channels (Tyerman 1992). More recently,

Pei et al. (1996) have identified a vacuolar Cl^- channel (VCL) in *Vicia faba* guard cells which is activated by a CDPK in the presence of ATP and Ca^{2+} and, to a weaker extent (22%), by protein kinase A. The VCL channel was activated at physiological potentials enabling Cl^- uptake into vacuoles. CDPK-activated channels were also observed in red beet vacuoles suggesting that these channels may be present ubiquitously in plant vacuoles. As mentioned above, this channel was also permeable to malate.

Nitrate—Nitrate is usually the anion which exhibits the highest permeability through the vacuolar membrane (Sze 1995). Due to the lack of appropriate radiolabeled nitrate, our knowledge of nitrate transport is still limited. One approach to study nitrate uptake is to use $^{36}\text{ClO}_3^-$ as a suitable radioactive nitrate-analogue (Deane-Drummond and Glass 1982). Chodera and Briskin (1990) showed that ClO_3^- uptake was ATP-dependent and saturable in sealed tonoplast vesicles isolated from red beet. ClO_3^- uptake was driven by an inside positive membrane potential, but not by an artificially imposed pH gradient. The fact that NO_3^- strongly inhibited uptake of ClO_3^- has been taken as evidence that the two compounds are translocated into the vacuole by the same carrier. However, compartmentation analysis (Martinoia et al. 1981, Miller and Smith 1992) indicates that the membrane potential difference between the cytosol and the vacuolar lumen, which is reported to be 20–50 mV (Sze 1995), is not strong enough to explain the accumulation of nitrate within the vacuole. From these experiments and from some transport experiments (Blumwald and Poole 1985b, Schumaker and Sze 1987a), it was concluded that, in addition to or alternatively to a membrane potential driven nitrate transporter, a NO_3^-/H^+ antiporter is present on the tonoplast. Because these observations are in contrast to those made using ClO_3^- , further experiments are required to elucidate the accumulation of nitrate.

Retrieval of NO_3^- may be coupled with nitrate reduction, which would deplete the cytosolic pool of nitrate, thus increasing the gradient between the cytosol and the vacuole and allowing vacuolar NO_3^- to be exported quantitatively. Isolated barley vacuoles release NO_3^- faster than other anions (Martinoia et al. 1986), indicating that an export channel or transporter is present in the tonoplast.

Sulfate—The uptake of SO_4^{2-} and HPO_4^{2-} has rarely been investigated in detail. Using tonoplast vesicles, it has been shown repeatedly that these anions cross the tonoplast much more slowly than NO_3^- or Cl^- (e.g. Churchill and Sze 1984). Using barley mesophyll vacuoles Kaiser et al. (1989) showed that SO_4^{2-} uptake is stimulated by MgATP, resulting in a net accumulation inside the vacuole. In the presence of inhibitors of the tonoplast, H^+ -ATPase uptake was reduced to the non-energized level, indicating that energization was due to the activity of the vacuolar proton pump. Concentration-dependent sulfate transport has been reported to be saturable with either a K_m value of 3.8 mM (Dietz et al. 1992) and 2.2 mM (Mornet et al. 1997) respectively or biphasic with a saturable (K_m 0.5 mM)

and a linear component (Kaiser et al. 1989). By the use of different protein modifying agents, it was established that the dianions malate and sulfate cross the tonoplast by distinct carriers (Mornet et al. 1997).

Phosphate—Using ^{31}P NMR, it has been shown that in *Acer pseudoplatanus* cells, P_i concentrations in the cytosol are maintained at a constant level. P_i starvation leads to an efflux of P_i from the vacuole; in contrast an accumulation of P_i in the vacuole was observed after the addition of P_i to non-starved cells (Rebille et al. 1983). Uptake of HPO_4^{2-} into isolated vacuoles was hindered by the very low uptake rates observed which did not permit a detailed analysis. Interestingly, ATP-dependent HPO_4^{2-} uptake into isolated barley vacuoles was more than doubled after phosphate starvation (Mimura et al. 1990). Using vacuoles isolated from *Catharanthus* cell cultures, Massonneau et al. (2000) succeeded in characterizing in more detail the vacuolar phosphate transporter. NMR studies had shown that phosphate fluxes across the tonoplast of these cells were much faster in comparison to other cell types (Sakano et al. 1992). Indeed, vacuoles isolated from *Catharanthus* also showed an increased uptake rate. Phosphate uptake exhibited a low affinity (K_m about 5 mM). Uptake was strongly stimulated by ATP and PP_i , and inhibited by SCN^- , indicating that the membrane potential plays an important role in vacuolar phosphate accumulation. Interestingly, CrO_4^{2-} , which is known to be a strong inhibitor of SO_4^{2-} uptake at the plasma membrane, was also a strong inhibitor of vacuolar phosphate uptake. However, the fact that *Catharanthus* cells exhibit a much higher phosphate uptake activity and may even die in the presence of an excess of phosphate may indicate that, in these cells, a regulatory element of phosphate transport is missing, either at the plasma or the vacuolar membrane.

Uptake of inorganic cations

The membrane potential of the cytosol with respect to the vacuole is negative (20–40 mV). This fact implies that cations are excluded from the vacuole unless transport is coupled to an energy-dependent uptake mechanism.

Potassium—Several channels exhibiting potassium permeability have been described (for a detailed review see Allen and Sanders 1997). The first channel demonstrated for vacuolar membranes was the so-called SV (slow activating vacuolar) channel (Hedrich et al. 1986, Coyaud et al. 1987, Hedrich and Neher 1987, Allen and Sanders 1997). This channel is defined by its slow activation and by Ca^{2+} and calmodulin-induced K^+ and Ca^{2+} fluxes. Furthermore, it is modulated by reversible phosphorylation (Bethke and Jones 1997). Permeabilities for Na^+ have also been reported for this channel. Due to its gating properties, it could participate in the release of these cations from the vacuole under circumstances in which the Ca^{2+} concentrations are increased by a signal.

The FV (fast vacuolar) channel activates instantaneously in response to voltage changes (Hedrich and Neher 1987, Allen and Sanders 1997). In contrast to the SV-channel, the FV-

channel is inhibited at Ca^{2+} concentrations above 100–300 nM (Hedrich and Neher 1987, Allen and Sanders 1996). This channel may allow the release of K^+ at low Ca^{2+} concentrations. Furthermore, depending on the prevailing electrochemical potential difference, uptake of potassium into the vacuole may also take place. Allen and Sanders (1997) suggested that the FV channel may also function as a shunt conductance for the electrogenic H^+ -pumps facilitating the vacuolar acidification.

A third type of K^+ channel is the VK channel which is activated instantaneously but nevertheless can be distinguished from the FV channel (Ward and Schroeder 1994, Allen and Sanders 1996). It is voltage independent and fully activated at 5 μM Ca^{2+} . Finally, VK channels are activated at low cytosolic pH. In higher plants, VK-channels have so far been described only in guard cells, and it is thought that they facilitate the release of K^+ during stomatal closure.

However, in many cases, the vacuolar K^+ -concentration is similar to that observed in the cytosol. Given the cytosol negative electrical potential difference at the tonoplast, an active K^+ translocation mechanism has to be postulated. A K^+/H^+ antiport mechanism has been reported for tonoplast enriched fractions from zucchini, *Brassica napus* hypocotyls, and *Atriplex* (Scherer and Martiny-Byron 1985, Hassidim et al. 1990, Cooper et al. 1991). However, in these cases the vacuolar fractions may have contained contaminating membranes, so a more detailed experiment is required to elucidate the mechanism of energized potassium uptake into vacuoles.

An alternative mechanism for accumulating K^+ in the vacuole was suggested by Davies et al. (1992). Current analysis in response to PP_i , the substrate of the vacuolar H^+ -PPase, indicated that in addition to protons, this pump was also able to pump K^+ (Rea and Sanders 1987). However, attempts to directly measure PP_i dependent K^+ using purified PPase from pumpkin (Sato et al. 1994) or grapevine vesicles (Ros et al. 1995) failed. From these results, it is tempting to conclude that the PPase functions only as H^+ -pump.

Assuming a membrane potential of 30 mV, the vacuolar K^+ concentration would still be 20 mM at equilibrium if the cytosolic concentration is assumed to be about 100 mM. Due to the large size of the vacuole, more than 40% of the total potassium would still be present in the vacuole. For an effective retrieval of potassium, which in some cases is a limiting growth factor, an energized export mechanism should be postulated.

Sodium—The ability of plants to survive and grow in a saline environment involves an integration of intracellular and multicellular adaptive mechanisms. It is generally assumed that intracellular compartmentation is a prerequisite for salt tolerance (Läuchli and Epstein 1990). The fact that plant cells maintain a high K^+/Na^+ ratio in the cytoplasm even under conditions where Na^+ is the predominant cation in the cell, suggests that Na^+ is actively transported out of the cytoplasm into the vacuole.

Using short-term in vivo ^{23}Na NMR spectroscopy with barley roots, Fan et al. (1989) observed that accumulation of

Na^+ was accompanied by vacuolar alkalization. Direct evidence for a Na^+/H^+ antiport was presented using tonoplast vesicles. Addition of Na^+ reversed the ATP-dependent acidification (Niemietz and Willenbrink 1985, Blumwald and Poole 1985a, Blumwald and Gelli 1997, Serrano et al. 1999). In some species the vacuolar Na^+/H^+ antiport activity appears to be constitutive, while in others, such as *Beta vulgaris* and barley the antiporter was activated in the presence of high Na^+ concentrations, although it is very likely that the transporter is constitutively expressed (Blumwald and Poole 1987, Garbarino and DuPont 1988).

Comparison of two *Plantago* species differing in their salt sensitivity showed that a Na^+/H^+ antiport could be detected only in vesicles from the salt tolerant species, but not from the more salt sensitive one (Maathius and Prins 1990). It therefore appears likely that the Na^+/H^+ antiport is a prerequisite for salt tolerance.

Recently, a vacuolar Na^+/H^+ antiporter of *Saccharomyces cerevisiae* has been identified (Nass et al. 1997). Using this sequence and the *Arabidopsis* data base, Apse (1999) isolated a plant homologue. Cloning and expression in *Arabidopsis* showed that this homologue indeed encodes for a vacuolar Na^+/H^+ antiporter. Interestingly, *Arabidopsis* overexpressing the Na^+/H^+ antiporter was more salt tolerant. Whether the increased salt tolerance is directly due to a higher antiport activity (the maximal accumulation of Na^+ is limited by the ΔpH) or to an increased vesiculation, as discussed by Frommer et al. (1999), has to be elucidated.

Calcium—It is well established that calcium plays a central role in signal transduction (e.g. Hepler and Wayne 1985). Its cytosolic concentration is far below 1 μM , and it reaches this concentration only during a calcium-dependent signaling. Far higher concentrations are observed in the apoplast and within the vacuole. An energized, highly specific calcium uptake by the vacuole is, therefore, a prerequisite for maintaining a low cytosolic calcium concentration.

P-type Ca-ATPases have been identified at the plasma membrane, the ER, and the vacuolar membrane (Chanson 1993, Askerlund 1997, Malmström et al. 2000, for a review see Geisler et al. 2000). These pumps exhibit a high affinity for Ca^{2+} (K_m 30 nM). Interestingly, transient expression of the vacuolar Ca-ATPase in *Arabidopsis* protoplasts indicates that this pump is localized on small vacuoles but not on the large central one (Geisler et al. 2000). In addition to the Ca^{2+} pump, a $\text{Ca}^{2+}/\text{H}^+$ antiporter has been demonstrated in vacuolar membrane fractions (Schumaker and Sze 1985, Schumaker and Sze 1986, Blumwald and Poole 1986). This antiporter exhibits a far lower affinity than the Ca^{2+} -ATPase (14–60 μM). Calmodulin stimulates $\text{Ca}^{2+}/\text{H}^+$ exchange, mainly at non-saturating Ca^{2+} concentrations. Both the vacuolar pH and calmodulin have been shown to decrease the apparent K_m of the $\text{Ca}^{2+}/\text{H}^+$ antiporter (Blumwald and Poole 1986, Andreev et al. 1990). Different stoichiometries between H^+ and Ca^{2+} have been reported (for a review see Blumwald and Gelli 1997); how-

ever, it should be mentioned that free Ca^{2+} concentrations in the vacuole may exceed 1 mM and that therefore a more than 1,000-fold Ca^{2+} -gradient has to be maintained between the cytosol and the vacuole. Assuming a ΔpH of 2 units between the cytosol and the vacuole, a stoichiometry of at least 2H^+ per Ca^{2+} has to be postulated. Because the pH difference is lower than this in many plants, $3\text{H}^+/\text{Ca}^{2+}$ would be often necessary in order to allow calcium influx. Blackford et al. (1990) have shown that clamping tonoplast vesicles at a constant pH results in an accumulation of Ca^{2+} which increases as the membrane potential is shifted to more negative (cytosolic) values. These results provide indirect evidence that, under physiological conditions, a Ca^{2+} ion is exchanged with three or more protons.

An important step towards a detailed understanding of the function of the vacuolar $\text{Ca}^{2+}/\text{H}^+$ antiporter has recently been reported. Two *Arabidopsis* genes (CAX1 and CAX2) that complement a *S. cerevisiae* mutant defective in vacuolar Ca^{2+} accumulation have been identified (Hirschi et al. 1996). Both gene products, which are structurally similar to microbial $\text{Ca}^{2+}/\text{H}^+$ antiporters, are able to catalyze a ΔpH dependent Ca^{2+} uptake. CAX1 was highly expressed in response to exogenous Ca^{2+} . Transgenic tobacco plants expressing CAX1 displayed symptoms of Ca^{2+} deficiency, including hypersensitivity to ion imbalances such as increased magnesium and potassium concentrations and to cold shock; however, increasing the Ca^{2+} in the media decreased these sensitivities (Hirschi 1999). Tobacco plants expressing CAX1 exhibited increased Ca^{2+} accumulation and altered activity of the tonoplast-enriched $\text{Ca}^{2+}/\text{H}^+$ antiporter. These results strongly suggest that regulated expression of the $\text{Ca}^{2+}/\text{H}^+$ antiporter is required for normal growth. A $\text{Ca}^{2+}/\text{H}^+$ antiporter from mung bean has recently been cloned and described kinetically (Ueko-Nakanishi et al. 1999). Using a GFP- $\text{Ca}^{2+}/\text{H}^+$ antiporter fusion constructs and classical fractionation studies Ueko-Nakanishi et al. (2000) could show that this antiporter is indeed localized in the vacuolar membrane of the central vacuole as well as in small vesicles which may correspond either to prevacuoles or small vacuoles. Furthermore, treatment of vacuolar membranes with a crosslinker revealed a band corresponding to the dimer (84 kDa) of the $\text{Ca}^{2+}/\text{H}^+$ antiporter, strongly suggesting that the transporter functions as a dimer in vivo.

In addition to the Ca-pump and $\text{Ca}^{2+}/\text{H}^+$ antiporter several vacuolar channels have been shown to be permeable to Ca^{2+} . The fact that several Ca^{2+} channels exist on the vacuolar membrane may indicate that the vacuolar calcium store is used to increase the cytosolic calcium concentration by different signaling pathways. However, it should be remembered that plant cells contain several types of vacuoles which can usually not be distinguished if tonoplast vesicles are isolated. Careful localization studies are needed to address this problem.

As mentioned above, the SV channel exhibits a K^+ as well as a Ca^{2+} -dependent current. Additionally, a vacuolar voltage-gated Ca^{2+} channel (VVCa) has been reported which is activated on membrane hyperpolarization (Johannes et al. 1992, Al-

len and Sanders 1994, Allen and Sanders 1997). While the effect of cytosolic Ca^{2+} is still a matter of debate, all studies report activation of the channel as vacuolar Ca^{2+} increases over the mM range. The function of the VVCa is probably to release Ca^{2+} if a signal affects the membrane potential difference between the cytosol and the vacuole.

Calcium selective channels which are activated after depolarization have also been described (Pantoja et al. 1992b, Allen and Sanders 1997). These channels could therefore mediate calcium influx into the vacuole. However, it is as yet unknown, whether the concentration ratios of calcium between the cytosol and vacuole would allow such a transport.

As in animals, several studies in plants have shown that addition of inositol 1,4,5-trisphosphate (IP_3) causes a specific and saturable transient release of Ca^{2+} from tonoplast vesicles or intact vacuoles (Schumaker and Sze 1987b). Inhibition of the IP_3 dependent Ca^{2+} release by TMB 8 or heparin (Brosnan and Sanders 1990) suggests a similarity between the mechanisms in animal and plants, despite the fact that the channels are localized in different membranes. Using the patch clamp technique, it has been shown that IP_3 dependent Ca^{2+} release from vacuoles is mediated by an IP_3 voltage-dependent calcium channel (Alexandre et al. 1990, Allen and Sanders 1994, Allen and Sanders 1997). Currents are inwardly rectifying over physiological negative membrane potentials. Half maximal opening was observed at 0.2 mM IP_3 . The high specificity of this channel for IP_3 indicates that this channel is implicated in calcium release during a signal transduction pathway; however, until now the stimuli able to activate the IP_3 -dependent channel remain unknown.

Magnesium—The presence of a $\text{Mg}^{2+}/\text{H}^+$ antiporter has been described for the vacuole-like lutoids of *Hevea brasiliensis* (Amalou et al. 1992) and tonoplast vesicles isolated from maize roots (Pfeiffer and Hager 1993). The partially purified and reconstituted transporter displayed a $\text{Mg}^{2+}/\text{H}^+$ antiport with a cation selectivity of $\text{Zn}^{2+} > \text{Cd}^{2+} > \text{Mg}^{2+}$, while Ca^{2+} was unable to dissipate an established pH gradient. These results indicate that, at least in the lutoid membranes, two different antiporters mediate the uptake of Mg^{2+} and Ca^{2+} .

Heavy metals—Plants need some heavy metals such as Cu^{2+} or Zn^{2+} as micronutrients. Therefore, they need transporters which enable them to take up these potentially toxic compounds. However, plants that grow in soils contaminated with heavy metals risk taking up toxic amounts of these metals. Some of these heavy metals are readily bound to the cell wall. A large portion of the heavy metals absorbed by the cell is usually accumulated within the vacuole. A vacuolar $\text{Cd}^{2+}/\text{H}^+$ antiport activity has been demonstrated (Salt and Wagner 1993). It will be interesting to investigate whether this antiporter corresponds to the $\text{Ca}^{2+}/\text{H}^+$ antiporter already identified. However, it is known that plants form chelates with heavy metals by synthesizing phytochelatin (PCs), and it has been shown that phytochelatin can be transported into vacuoles of *Schizosaccharomyces* as apoPC or as PC-Cd complexes by ABC transporters

(Ortiz et al. 1992, Ortiz et al. 1995). Vacuoles of higher plants are also able to transport phytochelatins (Salt and Rauser 1995, Tommasini, Grill and Martinoia unpublished); however, in this case, the stoichiometry between PC and cadmium has not been demonstrated. Furthermore, cadmium may also be transported by ABC transporters as bisglutathione-cadmium complex (Li et al. 1997a, Li et al. 1997b). Whether all these mechanisms work together in vivo is still unknown. In a recent review, Rea et al. (1998) discussed in detail the current knowledge of the role of ABC transporters in cadmium tolerance. Less is known about the transport of other heavy metals. It is assumed that Zn^{2+} is absorbed into the vacuole by a proton antiport mechanism and forms chelates with citrate within the vacuole. A comparative uptake study for zinc in tonoplasts of zinc sensitive and zinc tolerant *Silene vulgaris* revealed different properties, which, are however, difficult to interpret (Chardonens et al. 1999). *Arabidopsis* overexpressing an *Arabidopsis* gene related to a putative zinc transporter of mammals exhibited enhanced zinc tolerance (Van der Zaal et al. 1999). In contrast, antisense plants were more susceptible to zinc treatment. These results indicate that the corresponding gene product is indeed a zinc transporter and that it is probably localized on the vacuolar membrane.

Products of the secondary metabolism and xenobiotics— involvement of secondary energized transporters and directly energized, ABC-type transporters

Plants synthesize an enormous number of secondary metabolites and many of these have been found to be exclusively localized in the vacuole (reviews: Matile 1987, Wink 1997). Furthermore, during recent years, increasing evidence has indicated that modified xenobiotics are accumulated within the vacuole. It is not within the scope of this minireview to detail the transport processes involved in vacuolar accumulation of these diverse compounds (for a review see Wink 1997, Martinoia et al. 2000), but we wish to mention some general strategies.

Until 1993, it was generally assumed that accumulation of solutes in plants was due exclusively to the proton motive force. In this case, the electrochemical gradient established by the two vacuolar proton pumps is used by so-called secondary energized transporters as a source of energy (see also Fig. 1). It was demonstrated that the ΔpH was essential for the uptake of a number of phenolics, such as esculin (Werner and Matile 1985), *o*-coumaric acid glucoside (Rataboul et al. 1985), apigenin-7-(6-*O*-malonyl)glucoside (Matern et al. 1983), and anthocyanins from carrot (Hopp and Seitz 1987). Furthermore, trap mechanisms for phenolics (conformational changes) or alkaloids (ion trap) were postulated. Recently it became evident that in addition to transporters depending on the protonmotive force, directly energized transporters are present on the vacuolar membrane (Fig. 2). The first demonstration for a directly activated transport of solutes into the vacuole was provided for glutathione conjugates (Martinoia et al. 1993). It is well estab-

lished that a large number of xenobiotics are detoxified by glutathionation (Kreuz et al. 1996).

Presently, glutathione conjugated to endogenous secondary plant products, especially derivatives of the phenylpropanoid pathway like cinnamic acids or anthocyanins, are reported in plants (for review see Marrs 1996). In the case of anthocyanins in maize, it was shown that the last genetically defined step of anthocyanin synthesis is encoded by Bronze2 (Bz2) (Marrs et al. 1995). The gene product of Bz2 exhibited strong homology with glutathione *S*-transferases and was able to transfer glutathione to the artificial substrate CDNB (1-chloro-2,4-dinitrobenzene). It is therefore tempting to speculate that glutathionation of anthocyanins is a prerequisite for vacuolar import, because glutathione conjugates may be very efficient substrates for subsequent vacuolar deposition via ATP-driven ABC transporters. A subsequent study showed that the anthocyanin9 gene (an9) involved in anthocyanin synthesis in *Petunia* also exhibits homology with glutathione *S*-transferases and that an9 and Bz2 can functionally complement each other in *Zea mays* and *Petunia*, respectively, although these genes are only distantly related and belong to different subfamilies of glutathione transferases (Alfenito et al. 1998). However, up to the present it has not been possible to demonstrate that the Bz2 gene product is indeed able to transfer a glutathione moiety to anthocyanins (for more details see Martinoia et al. 2000).

A further hint that pterocarpanes may be glutathionated in vivo was presented by Li et al. (1997b) who showed that a maize GST is able to glutathionate medicarpin.

Directly energized transporters may nevertheless play a role in the accumulation of secondary plant compounds. Studies using isolated rye vacuoles, which contain flavonoid glucuronides, showed that these secondary plant compounds are transported by directly energized transport processes (Klein et al. 2000). Furthermore, studies with lucifer yellow, a sulfonated compound also indicates that sulfonated and sulfated secondary compounds cross the tonoplast by direct energization (Klein et al. 1997). The situation is less clear for glucosides. Comparing the vacuolar uptake of isovitexin, a glucosylated flavonoid occurring in barley and hydroxyprimisulfuronglucoside, a detoxification product of the herbicide primisulfuron showed that the two glucosides are transported by different mechanisms (Klein et al. 1996). While isovitexin was taken up by a proton antiport mechanism, the uptake of hydroxyprimisulfuron glucoside was directly ATP-dependent. Recent results have indicated that the same compound can be recognized by different transporters in different organisms. Comparison of saponarin (the main flavonoid of barley) uptake in barley and *Arabidopsis*, vacuoles indicates that this compound is taken up by a proton antiport mechanism in barley and by direct energization in *Arabidopsis* which does not contain this flavonoid (Frangne, Eggmann, Weissenböck, Martinoia, Klein; in preparation). From these results, it is tempting to speculate that glucosides are taken up by an antiport mechanism if structural an-

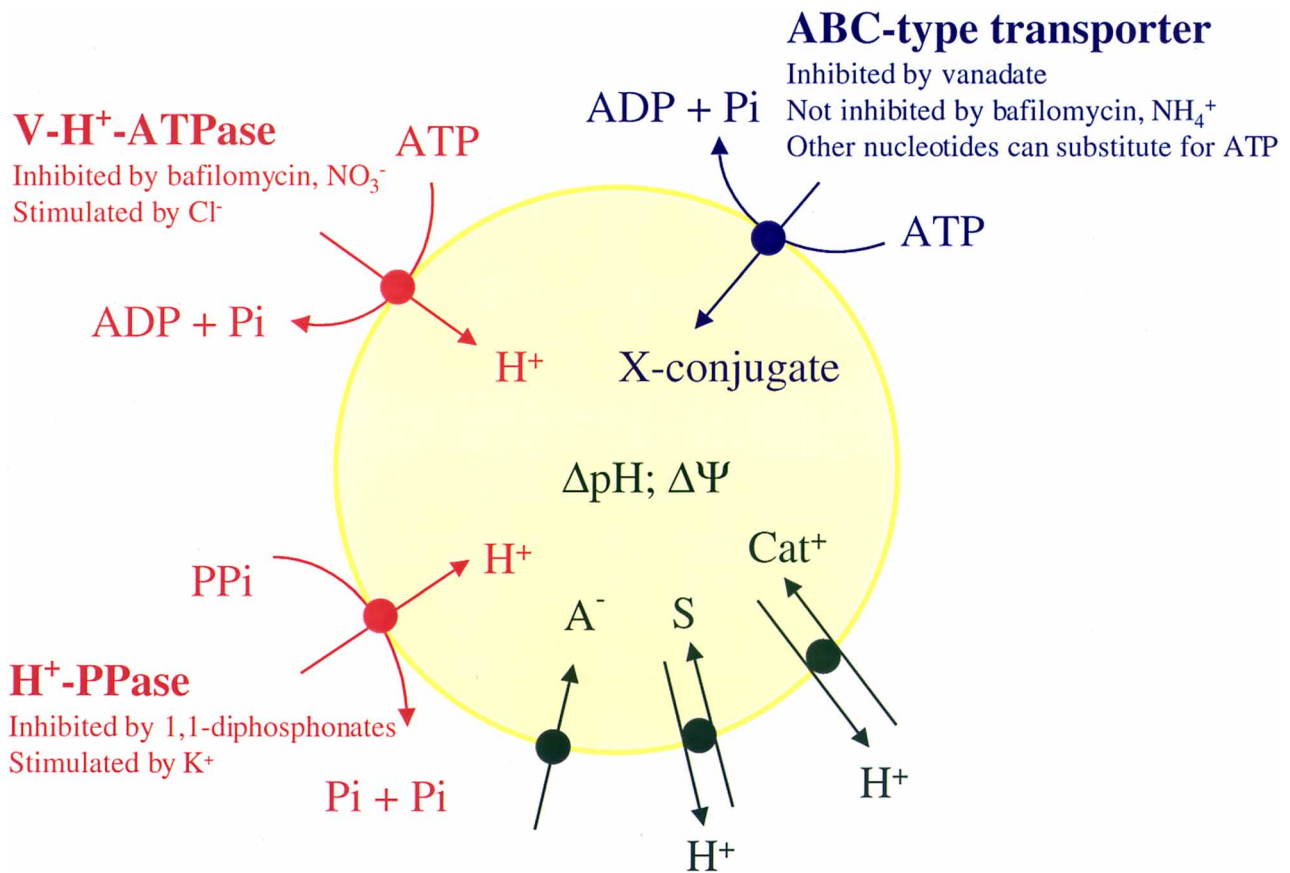


Fig. 1 Proton pumps establishing an electrochemical gradient (red), secondary energized uptake mechanisms (green), and directly energized, ABC-type transporters (blue) of the plant vacuole. S, neutral solute; A⁻, anion; cat⁺, cation; X-conjugate, conjugate of a compound X (secondary metabolite or xenobiotic) with a hydrophilic compound such as glucose, glutathione, an amino acid, malonate, or sulfate (for more details on ABC transporters see also Chapter 7 and Fig. 2).

alogues are present in the plant (or cell), while glucosides which have no structural analogues in the plant are transported into the vacuole using a directly energized transporter. However, this hypothesis must be proven using a large number of glucosylated compounds.

Leaf senescence is accompanied by an extensive loss of plastidic stroma proteins and breakdown of thylakoid membranes. During this phase, the thylakoidal pigment-protein complex is disassembled and, particularly, chlorophyll is broken down into phytol, Mg²⁺, and water-soluble cleavage products of the porphyrin moiety occurs (Matile et al. 1999). It has been shown that these cleavage products are removed and stored in the vacuole. In several cases, they have been found to be malonylated or glycosylated, modifications observed for many secondary metabolite such as flavonoids and anthocyanins. Efficient export of tetrapyrroles from metabolically active compartments is obviously necessary because of the toxicity of free tetrapyrroles when exposed to light. *Bn-NCC1*, a major chlorophyll catabolite of *Brassica napus*, is taken up in

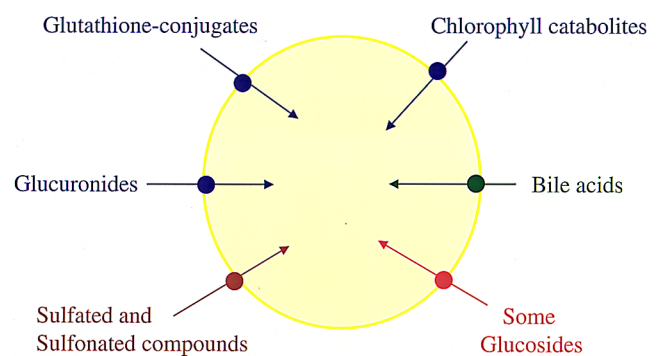


Fig. 2 Substrates transported into plant vacuoles by direct energization. Blue, Substrates for which an ABC transporter has already been identified. For a review on directly energized, ABC-type transporters see Rea et al. 1998, Rea 1999, and Martinoia et al. 2000.

barley mesophyll vacuoles by direct energisation (Hinder et al. 1996).

It is now well established that directly energized transport

of solutes occurs by ABC-type transporters. These transporters have attracted much interest, and several review articles have been published during recent years (Rea et al. 1998, Rea 1999, Theodoulou 2000, Martinoia et al. 2000). ABC transporters are found in bacteria, fungi, animals, and plants (Higgins 1992, Rea 1999, Martinoia et al. 2000). From the genomic sequencing project, it can be estimated that about 50 members of this family exist in Arabidopsis. In plants, four members of this family have been described in detail (Sidler et al. 1998, Lu et al. 1997, Lu et al. 1998, Tommasini et al. 1998). Three members belonging to the subfamily of the MRPs (multidrug related proteins); AtMRP1, AtMRP2, and AtMRP3 have been shown to be glutathione conjugate transporters (Lu et al. 1997, Lu et al. 1998, Tommasini et al. 1998). Interestingly, AtMRP2 and AtMRP3 are also able to transport chlorophyll catabolites produced during senescence and AtMRP3 can partially restore cadmium tolerance to *yef1*, a yeast mutant deleted in an ABC transporter and highly sensitive to cadmium.

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