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Review

Transporters in fruit vacuoles

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Abstract The vacuole is by far the largest organelle in fruits and can occupy more than 90 percent of the cell volume. Therefore, if we eat fruits and their products, we mainly eat the compounds stored within the vacuole. The main compounds are sugars, organic acids and secondary metabolites, such as phenolics and terpenoids, that are important for fruit quality. High concentrations of sugars, organic acids and inorganic ions in fruits generate a high osmotic pressure leading to a strong negative water potential that attracts water, allowing the fruit to grow. Accumulation of solutes within the vacuole requires many transporters in the vacuolar membrane, which is also called tonoplast. This review summarizes studies of transporters in fruit vacuoles, including proton pumps, aquaporins, sugar transporters, organic acid transporters and ABC transporters.

Key words: Aquaporin, proton pump, sugar transporter, vacuole.

Cell division in fleshy fruits, such as tomato and pear, occurs only in the very early stage of fruit development and fruits enlarge enormously only by cell expansion (Bain 1961; Yamaki and Matsuda 1977; Teitel et al. 1985; Bohner and Bangerth 1988). Most of the cell volume in fruits is occupied by a large central vacuole (Figure 1). Therefore, fruit growth and cell expansion depend on enlargement of vacuoles and increase in cytosolic space rather than cell division. The vacuole is the most important organelle for fruit quality because of its large size, and because compounds responsible for the taste and flavors of fruits, such as sugars, organic acids and secondary metabolites, are all within the vacuole and can be present at extremely high concentrations. Some fruits accumulate sugars at concentrations of almost 1 M at their mature stage (Whiting 1970). Organic acids in lemon fruits can be present up to 200 mM (Ulrich 1970). Secondary compounds are responsible for the typical taste of fruits, which in most cases is a blend between many phenolics and terpenoids. Some of these secondary compounds are at concentrations in the mM range, others are only at very low concentrations. Each of these compounds is believed to be transported into the vacuole by a specific transporter. Therefore, many transporters are required during fruit development and maturation. To facilitate water entry into the expanding fruit, so-called aquaporins are also required. Figure 2 summarizes representative transporters in vacuoles, including proton pumps, aquaporins, sugar, organic acid, secondary metabolite transporters. Maeshima (2001) and Martinoia et al. (2000, 2007) have reviewed vacuolar transporters. Therefore, in this review, we focus on transport processes specific to the fruit vacuole.

Acidity in fruits

Young, developing fruits are extremely acidic and accumulate mainly organic acids, such as malic, citric or tartaric acid. The pH of immature fruits is often below pH 3, but generally increases during fruit maturation.

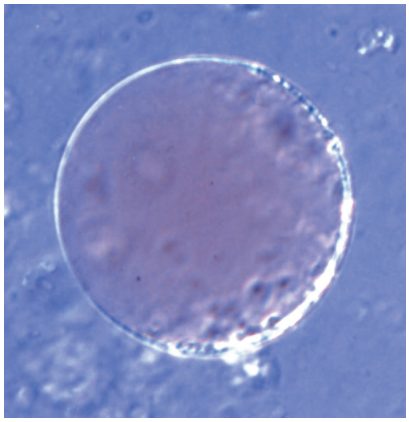

Figure 1. Protoplast of pear fruit. Protoplasts were prepared from pear fruit at 100 days after full bloom. The vacuole was stained by neutral red. A large central vacuole occupies most of the cell space. The picture is modified from Shiratake et al. (1998).

Abbreviations: ABC transporter, ATP-binding cassette transporter; MATE, multidrug and toxin extrusion transporters; PIP, plasma membrane intrinsic protein; TIP, tonoplast intrinsic protein; V-ATPase, vacuolar H^+-ATPase; V-PPase, H^+-pyrophosphatase.
This article can be found at http://www.jspcmb.jp/

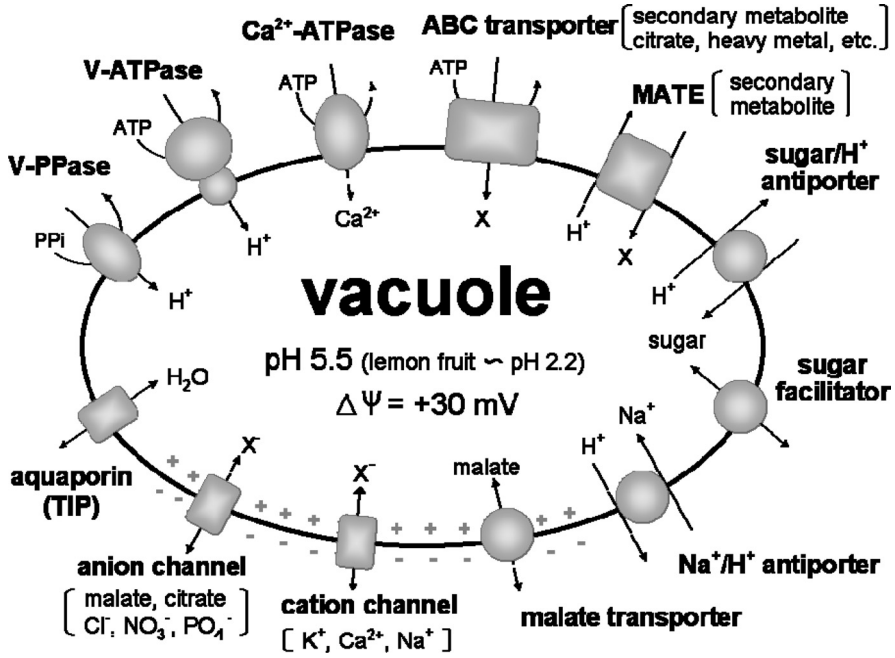

Figure 2. Transporters in vacuolar membrane. Proton pumps V-ATPase and V-PPase generate an electrochemical gradient (pH gradient and $\Delta\Psi$) across the vacuolar membrane. This gradient is required as a driving force for secondary energy-dependent transporters. Antiporters of sugar and Na^+, and MATE for secondary metabolite transport use a proton gradient for the driving force. Malate transporter and some anion and cation channels use the membrane potential difference ($\Delta\Psi$) as a driving force. Ca^{2+}-ATPase and ABC transporter transport substrates actively using the energy generated by cleavage of ATP. The ABC transporters transport many different molecules, including citrate, secondary metabolites, glutathione S-conjugates and complex heavy metals. Sugars are considered to be transported either by a sugar facilitator or a sugar/H^+ antiporter. Water is transported by aquaporins in the vacuolar membrane (TIP) depending on the water potential.

However, in many cases the pH remains low. Fruits do not taste acidic because large amounts of sugars accumulate during maturation. For example, many mature grape berries still have a pH of about 3.5, but they taste sweet because of the high amount of sugar accumulation (Terrier et al. 2001). The low pH of fruits is the result of two processes: 1) pumping protons into the vacuole, which directly results in a drop of pH, and 2) synthesis and accumulation of organic acids within the vacuole to serve as a buffer to maintain a low pH.

Proton pump

Two distinct proton pumps are in the vacuolar membrane: vacuolar H^+-ATPase (V-ATPase) and H^+-pyrophosphatase (V-PPase). They transport protons from the cytosol to the vacuole by cleaving energy-rich phosphate bonds of ATP and pyrophosphate, respectively. The electrochemical gradient across the vacuolar membrane generated by this process is essential as a motive force for secondary energy-dependent transporters, including sugar transporters, organic acid transporters, inorganic ion transporters and secondary metabolite transporters (Figure 2). V-PPase consists of a single kind of polypeptide, and V-ATPase is a multi-subunit enzyme complex comprising more than ten different subunits. Maeshima (2000), Ratajczak (2000) and Sze et al. (2002) summarize the structures, biochemical characters, regulations and functions of V-ATPase and V-PPase.

V-ATP and V-PPase have been purified from the vacuolar membrane of pear fruits (Hosaka et al. 1994; Suzuki et al. 1999a). The characteristics of V-ATP and V-PPase from pear fruits, such as subunit compositions and inhibitor sensitivities, are similar to those described for other plants. Interestingly, lemon fruits have two types of V-ATPase: a normal type expressed in fruits and other organs and has characteristics similar to V-ATPase from other plant species, and a fruit-specific type that has unique properties (Müller et al. 1996, 1997). The fruit-specific V-ATPase retains vanadate sensitivity, but the normal type does not. The H^+/ATP stoichiometries differ between the two types of V-ATPase: the normal type has an H^+/ATP stoichiometry of 2, and the fruit-specific type has a stochiometry of 1 (Müller et al. 1999; Müller and Taiz 2002). The pH of lemon fruits can reach 2.2 and therefore lemon fruit vacuoles are much more acidic than vacuoles from other organs and most other fruits. A lower H^+/ATP stoichiometry of the fruit-specific type of V-ATPase allows a steeper pH gradient between the cytosol and the vacuole. Terrier et al. (1998, 2001) characterized V-ATPase and V-PPase activities in the vacuolar membrane vesicles from grape berries. Interestingly, even though these fruits are strongly acidic, the V-PPase appears to be the major proton pump. Interestingly, the grape berry V-PPase has a temperature optimum above 50°C. Such a high temperature tolerance

may be an adaptive response by the grape vacuolar membrane, because the temperature of the surface of grape berries can reach 50°C.

Genes encoding V-ATPase subunits and V-PPase have been cloned from different fruit species and their expressions in fruit development have been determined, for example, V-ATPase subunit genes of pear (Amemiya et al. 2005b), Japanese pear (Suzuki et al. 2000), citrus (Takanokura et al. 1998), peach (Etienne et al. 2002) and tomato (Amemiya et al. 2006; Bageshwar et al. 2005; Coker et al. 2003), and V-PPase genes of pear (Suzuki et al. 1999b), Japanese pear (Suzuki et al. 2000), peach (Etienne et al. 2002) and grape berry (Terrier et al. 2001). The activity and protein levels of V-ATPase and V-PPase during fruit development have been determined also for pear (Shiratake et al. 1997a; Suzuki et al. 1999b), Japanese pear (Suzuki et al. 2000), grape berry (Terrier et al. 1998, 2001; Shiratake et al. 2001a) and tomato (Milner et al. 1995; Coker et al. 2003; Bageshwar et al. 2005). Changes in gene expression, protein level and activity of V-ATPase and V-PPase in fruit development differ between different fruit species and between different experiments, and they are somewhat confusing. V-ATPase and V-PPase may have different roles during fruit development (Shiratake et al. 1997a). In pear fruits, V-PPase activity is higher than V-ATPase activity soon after flowering, but V-PPase activity decreases markedly in the early developmental stage and V-ATPase activity increases with fruit growth. These results suggest that in pear fruits, as also suggested for other plants (Maeshima 2001), the V-PPase has a central role during the early stages of cell expansion and is more important for the generation of vacuoles in young fruits at the cell division stage. The V-ATPase increases with fruit growth and so contributes to fruit enlargement and to sugar and organic acid accumulation. Phytohormone treatment of pear fruit tissue changes gene expression, protein level and the activity of V-ATPase and V-PPase (Amemiya et al. 2005a), suggesting that phytohormones participate in regulating V-ATPase and V-PPase synthesis.

In peach fruit development, the expression pattern of genes implicated in organic acid metabolism (mitochondrial citrate synthase, cytosolic NAD-dependent malate dehydrogenase and cytosolic NADP-dependent isocitrate dehydrogenase) and genes of vacuolar proton pumps have been determined (Etienne et al. 2002). The expression pattern of vacuolar proton pumps is more consistent with the patterns of organic acid accumulation, suggesting that vacuolar proton pumps decide fruit acidity. Recently, antisense-transgenic tomato plants, in which the V-ATPase is suppressed specifically in fruits, have been produced (Amemiya et al. 2006). The transformed tomato plants grew normally, but they set smaller fruits and produced only few seeds

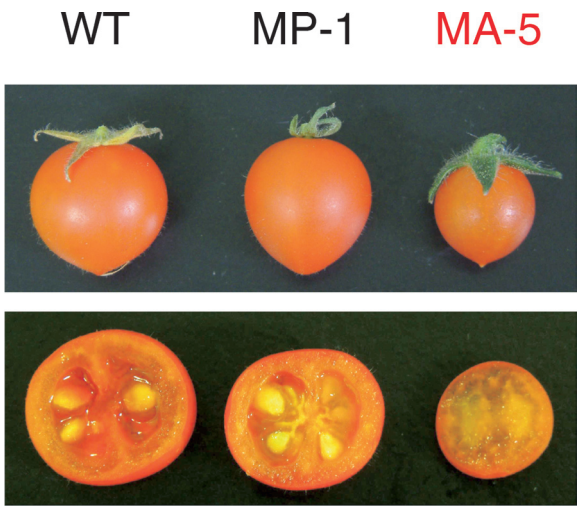

Figure 3. Fruit-specific suppression of V-ATPase in tomato. Fruits from a non-transformant (WT), a GUS control transformant (MP-1) and a V-ATPase A subunit-suppressed plant (MA-5). The V-ATPase-suppressed plant set smaller fruits, which contain few seeds. The figure is modified from Amemiya et al. (2006).

(Figure 3). Whether the production of a low number of seeds is a consequence of a reduced energy resource in smaller fruits or a direct effect of diminished V-ATPase activity has to be clarified in further experiments.

Organic acid transporter

Another factor determining acidity is the amount and type of organic acids. The main organic acids in fruits are malate (e.g. apple and pear) at pKs 3.4 and 5.1, citrate (citrus) at pKs 2.2, 4.8 and 6.4 and tartrate (e.g. grape berry) at pKs 3.0 and 4.4. Thus organic acids have a role as buffers and stabilize the pH. Also, each organic acid has a specific taste that contributes to the overall taste of fruits. Different transport systems have been described for malate, fumarate, tartrate and citrate. Using vacuolar membrane vesicles from tomato fruits, Oleski et al. (1987) showed that citrate enters the vacuole driven by the membrane potential, which could indicate that the citrate transporter corresponds to the malate channel (Martinoia et al. 2007). Uptake experiments at different pH suggested that citrate^{3-}, which is the predominant form in the cytosol, is transported. In contrast, Canel et al. (1995) described a directly ATP-driven transport process in citrus fruits, suggesting that an ATP binding cassette transporter (ABC transporter) drives the accumulation of citrate in citrus fruits. The contrasting transport mechanisms can be explained by different accumulation rates of citrate in tomato and citrus fruits. However, the acidic pH of citrus fruits converts citrate^{3-} to H_2citrate^{-}, which could act as a citrate trap. Therefore, identification of the corresponding transporters is necessary to understand better organic acid accumulation. A first step was made by identifying the malate transporter *At*tDT in Arabidopsis (Emmerlich et

al. 2003). Plants without this transporter accumulate much less malate. But transcript analysis suggested that this transporter, which does not correspond to the malate channel (Hurth et al. 2005), has also a role in vacuolar malate release. Indeed, a recent report by Shimada et al. (2006) provided evidence that a homologue of *At*tDT is implicated in citrate efflux in citrus fruits and hence is implicated in citrate homeostasis. A homologue of *At*tDT has also been identified in grape, suggesting that this transporter is implicated in malate and possibly tartrate transport in grape berry (Terrier et al. 1998). Malate channel and other components of organic acid transport is required to allow adjustment of organic acid composition and concentration in fruits.

Fruit acidity is a complex process that includes metabolic processes and vacuolar transport. Changing metabolic flows can result in pH differences. A recent attempt was made to integrate our present knowledge in a model of malate accumulation in fruits (Lobit et al. 2006). The model assumes that malic acid content is determined essentially by the conditions of its storage in the mesocarp cells. It was possible to predict the malic acid content of the fruit as a function of organic acids, potassium concentration, and temperature. The model was applied to peach fruit, and the predictions were in good agreement with experimental data.

Transport of water allows fruits to grow to a large size

Water is transported along the xylem and enters fruit cells because of a strongly negative water potential created by a high concentration of solutes in these cells. In the case of grape berries at the state of veraison, the xylem to the berries is interrupted and water enters the berries only along the phloem. Pear fruits show diurnal changes in fruit development (Shiratake, unpublished data), suggesting that water flows along the phloem. After water is transported along the xylem and the phloem, it moves into fruit cells and then into the vacuoles. Water crosses biological membranes mainly through specific water channels, the aquaporins.

Aquaporins

Aquaporin is also called water channel or major intrinsic protein (MIP). The aquaporin family is classified into four subfamilies based on their sequence similarity: plasma membrane intrinsic protein (PIP), tonoplast intrinsic protein (TIP), NOD26-like intrinsic protein (NIP) and small basic intrinsic protein (SIP) (Johanson et al. 2001). PIPs and TIPs are localized in the plasma membrane and the vacuolar membrane, respectively, and have been characterized in more detail than the other subfamilies. Their water transport activity was detected in the *Xenopus* oocyte expression system. The driving force for water transport along aquaporins across membranes is the water potential difference.

Water transport into fruit cells and vacuoles is indispensable for fruit development, and so aquaporins have a central role in the metabolism in fruit cells and in determining fruit size. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot of vacuolar membrane proteins from pear fruit showed that TIPs (γ- and δTIP) are the most abundant proteins (Shiratake et al. 1998), confirming the important role of facilitated water flow in fruit development. Expression patterns of TIPs differ between pear fruit, peach fruit and grape berry. In pear fruit, TIP protein and γTIP mRNA levels are especially high in young fruit and then decrease markedly, which correlates with the growth rates and hence water demand of pear fruits (Shiratake et al. 1997b, 2001b). In contrast, TIP protein levels increase gradually during grape berry growth (Shiratake et al. 2001a). This increase, even when fruits almost reach maximal size, might be explained by water transported only along the phloem in a later stage of grape berry development. The water potential difference between phloem and fruit cells is probably not as large as between xylem and fruit cells, and so water flow from the phloem to grape berry cells has to be facilitated as much as possible. In peach fruits, the mRNA level of γTIP is especially high in both the early stage and at the end of fruit growth and is lower between these stages (Sugaya et al. 2001). Peach fruit growth shows a double sigmoid curve, that is, the growth is high in the early stage, stagnant in the middle stage and is high again in the late stage. The mRNA levels of γTIP in peach fruit seems to be correlated with the fruit growth rate. In contrast to TIPs, changes in mRNA and protein levels of PIPs during fruit development are relatively small in apple (Hu et al. 2003), pear (Shiratake, unpublished data), grape (Shiratake et al. 2001a) and tomato (Werner et al. 2001). These results confirm that the vacuole has a central role during fruit cell expansion and that water has to cross the vacuolar membrane efficiently to allow rapid fruit growth. TIPs in fruit vacuoles are not regulated post-translationally, but PIP activity might be controlled by phosphorylation (Johansson et al. 1998). Consequently, only TIPs have to be tightly controlled transcriptionally, and a constitutive expression of PIPs still allows a control of water fluxes.

Chen et al. (2001) produced transgenic tomato plants of PIP, which they called tomato ripening-associated protein (TRAMP). Although over-expression of TRAMP has little effect on acid and sugar balance in fruits, suppression of TRAMP increases organic acids and decreases sugars. Whether PIP directly affects acid and sugar levels or deterioration of water flows by PIP suppression changes organic acid and sugar levels is not clear.

Recent studies suggest that some aquaporins can transport molecules other than water, such as CO_2, H_2O_2, glycerol, ammonia and boron (Tyerman et al. 2002). Speculating that CO_2 transport in fruits is facilitated by aquaporins is tempting, and determining if other compounds important for fruit quality are transported along aquaporins is necessary.

Importance of sugar for fruit taste and sweetness

The balance between acidity and sweetness is a major quality attribute. If a fruit is acidic and does not contain enough sugars, we sense it just as acidic, but if a fruit has too little acidity but a high concentration of sugars it is not tasty. Also for wines, if a grape berry contains too little sugars, the wine will have a low alcohol level and we sense this wine is just acidic. However, if too much malate has been degraded during grape berry maturation, but the sugar content is very high, we have the impression that the wine is less tasty. Some fruits, such as apple, citrus and grape, that accumulate high amounts of acids accumulate also a very high amount of sugars, mainly glucose, fructose and sucrose, which can reach nearly a molar concentration. Interesting is that in Roseceae trees, the main sugar transported within the phloem is the sugar alcohol sorbitol. But most Roseceae fruits, such as apple, pear, peach and cherry, readily convert most of this sorbitol to fructose. An exception is plums, which also convert sorbitol to fructose but still contain a high amount of sorbitol. Other sugars that accumulate in fruits are *myo*-inositol in kiwi and the C7 sugar alcohol perseitol in avocado. As in the case of organic acids, most of the sugars are stored within the vacuole and a strong sugar transport activity has to be assumed to fruit vacuoles, mainly during the ripening period. However, our knowledge of sugar transporters to fruit vacuoles is still limited.

Sugar transporters

Surprisingly, the sole energy-dependent sugar transport into fruit vacuoles reported so far to our knowledge is a sorbitol transporter in apple fruits (Yamaki 1987). However, sucrose uptake into vacuolar membrane vesicles of tomato fruits (Milner et al. 1995), hexose uptake into vacuolar membrane vesicles of pear fruits (Shiratake et al. 1997b), and sucrose and hexose uptake into vacuolar membrane vesicles of sweet lime juice-cells (Echeverria et al. 1997) are not promoted in the presence of ATP. Although both energy-dependent and energy-independent sugar transport activities have been detected also in vacuolar membranes from other plant organs (Martinoia et al. 2000, 2007), genes encoding vacuolar sugar transporters may not have been identified so far. However, two candidate genes are a hexose transporter in sugar beet (Chiou and Bush 1996) and a *myo*-inositol transporter in *Mesembryanthemum crystallinum* (Chauhan et al. 2000), but their sugar transport activity has not been described.

Recently, several proteomic analyses of vacuoles have been done to identify novel transporters in the vacuolar membranes of Arabidopsis (Carter et al. 2004; Sazuka et al. 2004; Shimaoka et al. 2004; Szponarski et al. 2004). These proteomic analyses identified some putative sugar transporters, but in most cases the correct locations of these candidates were not verified. A recent proteome analysis of barley mesophyll vacuolar membrane proteins identified a sucrose transporter homologue, HvSUT2 (Endler et al. 2006). A GFP fusion construct of HvSUT2 and its Arabidopsis homologue (AtSUT4) transiently expressed in Arabidopsis leaves and in onion epidermal cells was targeted to the vacuolar membrane (Endler et al. 2006). Whether this transporter corresponds to the sucrose importer, because the homologous gene products are proton co-transporters and hence drive sucrose export, has to be established in future experiments. However, when considering all vacuole proteomic data and the huge number of putative sugar transporters in the Arabidopsis genome, sugar uptake may be driven by a many transporters that might differ in timing of expression and substrate specificity.

Flavors and colors

Flavors make the real difference between fruits. The main compounds responsible for flavors are secondary metabolites, such as phenolics, mainly flavonoids, terpenoids and alkaloids. Flavonoids that contribute fruit colors are often anthocyanains. In fruits, transporters for these compounds may not have been reported. Genetic evidence showed that in maize ABC transporters of the multidrug resistance-associated protein (MRP) subfamily, together with glutathione transferases, are anthocyanin transporters and hence ABC transporters might be implicated in flower and fruit pigmentation. Another transporter family implicated in secondary metabolite transport is the multidrug and toxin efflux transporters (MATE). Genetic evidence suggests that this class of transporters are responsible for H^+-coupled flavonoid transport and possibly the transport of other phenolic compounds (Debeaujon et al. 2001). Speculating that these two classes of transporters are responsible for deposition of large amounts of phenolics in vacuoles is tempting. The problem of investigating ABC and MATE transporters is that many genes code for these classes of transporters and some redundancy must be expected. Secondary compounds responsible for flavors are often very plant specific and so, in contrast to organic acids and sugars, investigation of flavors requires a very plant-specific approach. Even less is known about

transport of terpenoids. However, ABC transporters of the pleiotropic drug resistance (PDR) subfamily of tobacco transport terpenoids, such as sclareol (Jasinski et al. 2001; Stukkens et al. 2005).

Conclusions

Despite the importance and uniqueness of fruit vacuoles, we know little about vacuolar functions and vacuolar transporters. Martinoia et al. (2000, 2007) and Maeshima (2001) summarizes vacuolar transport system of nitrate, phosphate, chloride, calcium, potassium, sodium, magnesium, heavy metals and some amino acids, but little information on fruit vacuoles has been reported. The function of fruit vacuoles differs between fruit species and changes during fruit development in each fruit. Although the main sugars and organic acids are similar in many fruits, secondary metabolites responsible for flavors are often unique. Proteomic analysis of highly purified fruit vacuoles might help to find new transporters responsible for fruit development and quality and to deepen our understanding of vacuolar functions in fruit.

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