Grape berry vacuole: a complex and heterogeneous membrane system specialized in the accumulation of solutes

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Abstract

Vacuoles fulfill highly specialized functions depending on cell type and tissue, and plant developmental stage. This complex and dynamic organelle is the main reservoir of grape berry cells, playing a major role during fruit development and ripening. Berry development is accompanied by modifications in size, composition, colour, texture, flavour, and pathogen susceptibility, mostly due to changes in vacuolar content. Most aroma and flavour compounds are not evenly distributed in the berry, and the number and type of vacuoles may vary depending on the tissue (skin, flesh and seeds). Together with the lytic (LV) and protein storage vacuoles (PSV) widely spread in plant cells, "phenolic vacuoles" are also implicated in cellular storage in grape cells. After *véraison*, when grape berry growth exclusively results from cell enlargement, tonoplast transporter proteins mediate a massive sugar import and water intake into the vacuole, which leads to a large vacuolar expansion. The pumps V-ATPase and V-PPase create a proton electrochemical gradient across the tonoplast, which, in turn, energizes the uptake of charged and uncharged solutes. Several tonoplast proteins mediating the uptake of sugars, organic acids, water, ions and anthocyanins have been cloned and some of them have been functionally characterized. The present review focuses on the storage function of vacuoles, as well as on their structure and diversity in relation to development and ripening of the grape berry.

Keywords: grape berry, vacuole, tonoplast transporters, sugars, organic acids, phenolics, aroma compounds.

1. Introduction

Grape (*Vitis vinifera* L.) is a major crop worldwide. The majority of fruit production is processed into wine, but significant portions are consumed fresh, dried into raisins, processed into non-alcoholic juice, and distilled into spirits (reviewed by Conde et al. 2007). Berry content at harvest is a major parameter for wine quality. The size and the quality of the berries mainly depend on water, sugars (glucose and fructose), organic acids (malic and tartaric acids), phenolic compounds (anthocyanidins and tannins) and aroma precursors.

The vacuoles are an essential organelle for plant cell physiology and therefore for plant life (Martinoia et al. 2007). They are responsible for the high cell surface-to-protoplasmic volume ratio required for extensive exchanges of material and information between plant cells and their environment. The vacuole is surrounded by a biological membrane called the tonoplast which separates the vacuolar lumen from the cytosol. Phospholipids, sterols, and ceramide monohexoside(s) are the major lipid classes in the tonoplast and plasma membrane, but the content of phospholipids on a protein basis is higher in the tonoplast (Yoshida and Uemura, 1986). Together with the cell wall, they control turgor, which is basic to cell hydraulic stiffness and plant growth (Marty 1999). Recognized functions of the vacuole also encompass storage (ions, metabolites, and proteins), digestion, pH and ion homeostasis, biotic and abiotic defence responses, toxic compound sequestration, and pigmentation (Marty 1999, Martinoia et al. 2000, Carter et al. 2004). The volume of the grape berry cells is largely occupied by a central vacuole. Besides its multifaceted roles, the vacuole of grape berry cells has attracted attention mainly because the storage function contributes directly to the quality of the fruit. Vacuoles are the main reservoir of grape berry cells for sugars, organic acids, aromas, flavours, ions and water, that are differently distributed throughout berry tissues (Figure 1).

Many scientific advances have led to an increased understanding of physiological, biochemical, and molecular aspects of grape berry maturation; however, little is known about the mechanisms, coordination, regulation and environmental sensitivity of the transport steps involved in vacuolar accumulation of solutes (Shiratake and Martinoia 2007). More generally, despite the importance and uniqueness of fruit vacuoles, the identity and functioning of vacuolar transporters still need further investigation at the molecular level (Maeshima 2001, Shimaoka et al. 2004, Carter et al. 2004, Endler et al. 2006).

The present review focuses on the diversity and biochemistry of the grape berry components, which contribute to the organoleptic properties of the fruit and wine, in relation with their vacuolar compartmentation during berry development and ripening. Emphasis will be given to the storage function of the vacuoles, which rely on the coordinated activity of transport proteins such as proton pumps and antiporters. Changes in the vacuolation degree, as well as vacuole morphological diversity will be related to the physiological role of this highly dynamic organelle during fruit development and ripening.

2. Structure and biochemical diversity of grape berry vacuoles

Plant vacuoles are highly dynamic, multifunctional organelles which provide the primary site of macromolecule storage and turnover. These organelles, that occupy as much as 90% of most mature cells, are an integral part of the endomembrane system, serving as the terminal products of the secretory pathway (Marty 1999). The space-filling function of the vacuole is essential for cell growth, because cell enlargement is accompanied by expansion of the vacuole rather than of the cytosol (Maeshima 2001). Ripe grape berry flesh cells are very heterogeneous, showing evident vacuolar diversity both in size (ranging from 1 to 50 μm of diameter) and content. Vacuoles in these cells vary from large and small colourless vacuoles to numerous small acidic vacuoles distributed throughout the cytoplasm, with a large central-vacuole also being observable in some cells (Figure 2). The diversity of vacuolar functions

parallels their diversity in morphology, biochemistry, and biogenesis (Marty 1999). As grape berries develop, the vacuolar system displays modifications in vacuole number, size, and in composition. As referred to above, fruit volumetric growth after *véraison* primarily results of vacuolar enlargement by incorporation of water and solute.

The complexity of the vacuolar system offers a rich field of future work for plant biologists. The vacuolar fraction from *Arabidopsis thaliana* shows the presence of many small vesicles attached to the main vacuole, whose intravesicular environment is also acidic as shown by neutral red staining (Shimaoka et al. 2004). Also, in specialized cells like fleshy cells of the grape berry, the vacuolar volume and content may vary widely (N. Fontes and co-workers, unpublished data, 2011). This diverse vacuolar morphology probably reflects the multiple roles of the vacuole system, as evidenced by many reports that emphasize the existence of different kinds of vacuoles in plants (Paris et al. 1996, Marty 1999, Bethke and Jones 2000). As early as 1876, from his observations on the anthocyanin vacuoles of *Drosera,* Charles Darwin documented that in a given tissue the shape, number and volume of vacuoles in a cell may vary (De 2000). Indeed, more than one kind of vacuole has been observed in cells undergoing maturation (Bethke and Jones 2000), where some vacuoles primarily function as storage organelles and other as lytic compartments (Paris et al. 1996, Marty 1999, Bethke and Jones 2000, Jiang et al. 2001, Martinoia et al. 2007).

Apart from the lytic (LV) and protein storage vacuoles (PSV), widely distributed in plant cells (Paris et al. 1996, Marty 1999, Jauh et al. 1999, Jiang et al. 2001, Reisen et al. 2005), “tannin vacuole”, “mucilage vacuole”, “lipid vacuole” and “phenolic vacuole” have been described (De 2000). Minorsky (2001) reported that the “phenolic vacuoles” were the so-called “specialized vacuoles”. The distinguishing features of phenolic vacuoles include their high phenolic content, avidity for some basic dyes (eg. neutral red), unusually acidic interior, great sap viscosity, and great refractivity (Minorsky 2001). The avidity of phenolic vacuoles for basic dyes is due to dye precipitation by endogenous phenols. Furthermore,

because tannins are, in general, amorphous astringent substances which combine with ferric salts to produce blue, black or green colour in sap, phenolic vacuoles have been named as "A" type or "full cell sap", in contrast to "B" type or "empty cell sap", which do not contain phenolics (De, 2000). Secondary metabolites are synthesized, degraded and stored by a series of integrated processes controlled mainly by membranes and by the different physico-chemical conditions present in the different cellular compartments. Because most of these metabolites are toxic to the plant itself, their vacuolar compartmentation may improve the efficiency of their production and avoid harmful effects in the cells (Roytrakul and Verpoorte 2007).

When attempting to study the vacuolar system of grape mesocarp cells, a precise identification mechanism of each compartment is needed. The tonoplast-intrinsic proteins (TIPs) are the most abundant vacuolar transporters (reviewed by Gomes et al. 2009), and several TIP genes, typically found in individual plant species, are differentially regulated, suggesting that different TIPs may be utilized under specific conditions (Bethke and Jones 2000). Jauh and co-workers (1999) showed that different kinds of vacuoles are labelled with different combinations of TIPs. As a result, they have proposed TIPs as markers of vacuole function and developmental stage (Jauh et al. 1999). However, it remains to be confirmed if these antibodies identify TIP orthologs in other species.

The use of fluorescent probes may also be useful in characterizing vacuole functions. However, vacuoles contain large amounts of anthocyanins and flavonols producing an intense autofluorescence, the emission of which varies according to local pH and ionic conditions. This may impair the utilization of fluorescent probes (Johnson 2006).

Many fine modifications in cell structure, especially those concerning the vacuolation degree, as well as vacuole morphological diversity, may result of environmental constraint and fruit maturation stage. At *véraison,* the large vacuole splits into smaller vacuoles generating a complex internal membrane structure. Changes in the vacuolation degree of

grape cells may be involved in the maintenance of turgor pressure or in the shift to storage and digestion, as reported for root apex cells of soybean (Klymchuk et al. 2003).

After *véraison*, the grape berry tissues, and thus the vacuolar content, change from full-scale defence against bird, insect and fungus, to an appealing sugary tissue, with reduced malic acid content. At this time, massive sugar accumulation makes the berry very attractive to birds and mammals and allows seed dispersion (Hardie et al. 1996). Most of the aroma and flavour compounds are not evenly distributed in the berry tissues, and their composition and concentration vary along with development and maturation. Accordingly, the vacuolation degree or type of vacuole may also depend on the berry tissue. Prior to ripening, tannins and polyphenols accumulate in outer mesocarp cells that surround the tissues of the peripheral vascular network, and later on during ripening they accumulate in greatest abundance in the exocarp cells. Besides storing tannin compounds, the "tannin rich cells", with "tannin vacuoles", may protect the vascular parenchyma cells against UV light. Also, these cells possess vacuoles acting as intermediate storage sites for the fluxes of assimilates that, as in other tissues, probably exit the phloem of the peripheral vascular network prior to their re-entry into the symplast of the pericarp parenchyma (Hardie et al.,1996).

3. The vacuole as a storage compartment

Major concerns for grape growers are the control of the ripening time, berry size and colour, acidity and the relative range of volatile and non-volatile aroma and flavour compounds. Therefore, understanding how and when various components accumulate in the berry, and how berry development and maturation responds to environmental stress factors, is of critical importance to adjusting grape growing practices and thus modifying wine typology (reviewed by Conde et al. 2007).

As already stated, grape berry vacuoles accumulate sugars, organic acids, aromas, flavours, ions and water (Figure 1). Each of these compounds is transported across the

tonoplast by a specific transporter protein that may be an active pump, a carrier or a channel. Some tonoplast transporter proteins have been identified and functionally characterized in grape cells (Figure 3), but solute compartmentation in the vacuoles of grapevine cells is still poorly documented.

Two proton pumps energize the vacuolar membrane

Grape berry vacuoles maintain an acidic pH, ranging from pH 2.5 in the green stage to pH 3.5 during ripening. The maintenance of ion and proton concentration gradients across the tonoplast membrane is essential for acid and sugar homeostasis in the berry (Hanana et al. 2007). The low pH of the vacuole of fruit cells is the result of two processes: i) proton pumping across the tonoplast, and ii) synthesis and accumulation of organic acids in the vacuolar sap (Shiratake and Martinoia 2007). Two distinct primary proton pumps, the vacuolar ATPase (V-ATPase) and the vacuolar inorganic pyrophosphatase (V-PPase), generate a proton electromotive force, which, in turn, allows the secondary active transport of inorganic ions, sugars and organic acids (Blumwald, 1987; Maeshima, 2001; Martinoia et al., 2007; Fontes et al. 2010b). The V-PPase is generally more active than the V-ATPase in young tissues with relatively high amounts of pyrophosphate (PPi) that is a by-product of biosynthetic pathways (Martinoia et al. 2007). However, the V-PPase is the predominant vacuolar proton pump in tonoplast vesicles from mature grape berries (Terrier et al., 1998) and intact vacuoles from CSB (Cabernet Sauvignon Berry) suspension cultured cells (Fontes et al. 2010b). Intact vacuoles are good experimental models to monitor the mechanisms of vacuolar acidification and solute uptake (Fontes et al. 2010b).

Vacuolar compartmentation of sugars in grape berry

Although the transport mechanisms of monosaccharides and disaccharides at the plasma membrane level are reasonably understood in several plants, including grapevine, information

on tonoplast sugar transporters is still limited. However, some tonoplast monosaccharide transporters (TMT) have been recently reported as mediating a proton-coupled antiport mechanism. Three AtTMT (*Arabidopsis thaliana* tonoplast monosaccharide transporters) isoforms were localized at the tonoplast by fusion with the green fluorescent protein (GFP) (Neuhaus 2007) and the tonoplast glucose/H^+ antiporter AtVGT1 (At3g03090) was characterized in the same plant model (Aluri and Büttner 2007). In *V. vinifera*, the hexose transporter VvHT6 is presumed to be targeted to the tonoplast (reviewed by Agasse et al. 2009). The sequence of VvHT6 is similar to that of the three AtTMT with an extended loop between the transmembrane helixes six and seven (Büttner 2007; Hayes *et al.* 2007), and its pattern of expression is consistent with a role in post-*véraison* import of hexoses into the vacuole. Uptake activities of the plasma membrane hexose transporters VvHT1, VvHT4 and VvHT5 have been demonstrated by heterologous expression in the hxt-null mutant yeast, but attempts to confirm the transport activity of VvHT6 has had little success (reviewed by Agasse et al. 2009).

Besides their role in sugar storage, vacuoles are also involved in the biosynthesis of higher saccharides from mono- or disaccharides. Also, vacuoles are likely the site for glycosylation and production of various metabolites (De 2000).

Water incorporation in the grape berry and the role of aquaporins

Vine water deficit has a clear implication in wine composition and sensory attributes (Roby et al. 2004). It generally leads to smaller berries (Bravdo et al. 1985, Kennedy et al. 2002, Matthews et al. 1990), thus increasing the skin to juice ratio, which, in turn, may increase the concentration of anthocyanins and phenolics in the must and wine (Reviewed by Conde et al. 2007).

Most of the berry volume gained before *véraison* is due to water import through the xylem, whereas most of the post-*véraison* gain is due to water import through the phloem.

This strong phloem component at *véraison* might explain the insensitivity of the berry to plant water deficits (Matthews et al. 1987). In addition, the shift of phloem unloading from symplastic to apoplastic pathway at *véraison* is associated with sugar accumulation at high levels in sink organs (Patrick 1997, Zhang et al. 2006), favouring the maintenance of a turgor pressure gradient. Moreover, the involvement of cell compartmentation of water and solutes makes more and more difficult for the leaves to extract water from ripening berries (Keller et al. 2006). The co-expression of some aquaporins and sugars transporters suggests a functional link between sugar and water fluxes during the processes of unloading and sugar accumulation in the vacuoles of the flesh cells (Delrot et al. 2001). Aquaporins are specialized proteins in the major intrinsic proteins (MIP) family that are implicated in water transport across biological membranes (Fouquet et al. 2008, Gomes et al. 2009).

Eight cDNAs encoding putative *Vitis* aquaporins (PIPs, Plasma Membrane Intrinsic Proteins and TIPs, Tonoplast Intrinsic Proteins) were found to be mostly expressed in roots, eventually enhancing and regulating water permeability (Baiges et al., 2001). The aquaporin VvPIP1A mediates water transport and is mainly expressed in the berries after *véraison* (Picaud et al., 2003). Moreover, after the release of the grapevine genome in 2007 (Jaillon et al., 2007), Fouquet and co-workers (2008) have identified 28 genes encoding putative aquaporins, and they have isolated 9 cDNAs encoding putative PIP and TIP aquaporins from grape berries at various developmental stages. Aquaporin gene expression is strongly regulated during berry development and globally decreases during ripening. The tonoplast aquaporin VvTIP2;1 and the plasma membrane aquaporin VvPIP2;1 are highly expressed in dividing and elongating cells and in cells involved in water and solutes transport (Fouquet et al. 2008). More recently, TIP2;1 was confirmed to transport water when individually expressed in *Xenopus* oocytes (Vandeleur et al., 2009).

Vacuolar compartmentation of malic and tartaric acids

The final organic acid content of the berries depends on the amount of acid synthesized, stored in the vacuole and degraded during the ripening stages. Organic acids are produced both in the leaves and fruits, but their biosynthetic mechanisms and compartmentation in the grape berry cells remain poorly understood. Along with berry development and ripening, the berry acid content varies. As reported above malic acid rapidly accumulates at early stages and decrease at the onset of ripening. Tartaric acid amount is kept constant and its concentration declines mainly due to dilution as berry volume increases.

During the vegetative growth phase, the sugars coming from photosynthesis in the leaves are transformed into malic acid which accumulates in the vacuoles of pericarp cells (Schulze et al. 2002). Also, it is believed that green berries are photosynthetically active and produce malic acid as a source of carbon and energy (Sweetman et al. 2009). Contrarily to many other fruits, grapes are incapable of storing significant amounts of starch. Malic acid is accumulated in the fleshy cells at the end of the berry's first growth phase and reaches a maximal value just prior to *véraison*. At *véraison*, due to the severe inhibition of the glycolytic pathway, malic acid import from the large central vacuole allows energy production, lowering grape malate levels. The decrease in malic acid in the grape berry at the onset of ripening also results from reduced malate synthesis, but the reduction in the amount of acid translocated from the leaves to the berries may also play a significant role.

Malate is accumulated in vacuoles at very high concentrations (> 300 mM) and the acid exchange across the tonoplast is believed to be driven by the electrochemical membrane potential difference (Martinoia et al. 2007). In Arabidopsis, malate exchange between the vacuole and the cytoplasm is mediated by AttDT, a tonoplast malate transporter (Emmerlich et al. 2003) and by AtALMT9, a tonoplast malate channel (Kovermann et al. 2007). Hurth et al. (2005) reported that the activity of AttDT is critical for pH homeostasis.

In grape berry, four good malate transporter candidates have been identified by blast analysis of the *Vitis vinifera* genome with the AtALMT9 protein sequence (Rongala 2008).

These genes are developmentally regulated. VvALMT9:1 and VvALMT9:2 showed post-*véraison* expression, while VvALMT9:3 is expressed at high levels before *véraison*, and VvALMT9:4 is poorly expressed. A single *AttDT* homologue has been identified in *V. vinifera* (Rongala 2008). An homologue of *AttDT* has been implicated in citrate efflux in citrus fruits (Shimada et al. 2006).

As already stated, the drop in tartaric acid content, from 150 mM at *véraison* to 25-75 mM at maturity, is mostly due to the increase of the berry size. Also, the free versus salt state of tartaric acid generally changes throughout maturation, contrarily to malic acid that generally remains as a free acid. Little is known about the biochemical mechanisms involved in tartaric acid accumulation in the vacuole of grape cells.

Vacuolar compartmentation of potassium

Several ions, such as potassium, calcium, magnesium and chloride are implicated in numerous physiological processes that impact fruit quality and, ultimately, wine taste and flavour. Therefore, the study of the biochemical steps involved in their compartmentation and interaction with other solutes is of major importance for plant physiologists and viticulturists.

Potassium is an essential macronutrient for grape berry growth and development, and a well-known enzyme activator. It is the main cation in must and wine (~ 900 mg/L; reviewed by Conde et al. 2007). Potassium is the main osmoticum in skin cells, as sugar is in the flesh. Because potassium may accumulate in the vacuole, it affects several transport processes across the tonoplast membrane. Thus, potassium uptake may increase the release of malic acid, favouring its metabolism in the cytoplasm (Jackson 2008, Davies et al. 2006).

Recently, two cDNAs encoding potassium plasma membrane transporters (VvKUP1 and VvKUP2) from grape berries were isolated and their function was demonstrated by heterologous expression in an *Escherichia coli* mutant deficient in potassium uptake (Davies et al. 2006). The two transporters are highly expressed in the berry skin during the first phase

of berry development, suggesting that, at this time, they play a role either in potassium uptake into the berries or in its compartmentation into the skin cells. However the transcript levels of both transporters are still significant at post-*véraison* suggesting that VvKUP1 and VvKUP2 may therefore continue to contribute to potassium homeostasis throughout berry repening. At the tonoplast level, a NHX-type cation/H^+ antiporter was recently cloned and functionally characterized (Hanana et al. 2007). VvNHX1 couples the passive movement of H^+ out of the vacuole to the active incorporation of monovalent cations (mainly K^+ and Na^+), playing an important role in vacuolar ion homeostasis in grape berries.

Vacuolar compartmentation of phenolic compounds

Phenolic compounds are an extended family of secondary metabolites in grape berries (Table 1). They are involved in plant protection because they are active growth inhibitors of other living systems. Also, they add colour and flavour to the fruit, contributing to the mouthfeel, quality and palatability of red wines. The major flavours associated with polyphenols are bitterness and astringency. Additionally, they may play important beneficial roles for human health as strong anti-oxidants (reviewed by Conde et al. 2007) and as activators of the human oestrogen receptor alpha (Chalopin et al. 2010). Many secondary metabolites, particularly phenolic compounds are frequently accumulated as glycosides, which increases their solubility, transport and storage ability (De 2000). Non-flavonoid phenolics accumulate primarily in the vacuoles of mesocarp cells, but flavonoids accumulate in the dermal cells of the skin tissue (Table 1).

Tannins or proanthocyanidins are polymers of flavan-3-ols and are the most abundant class of soluble polyphenolics in grape berries. A number of studies involving the identification of several enzymes, transcriptional regulators of proanthocyanidins biosynthesis and transporters have illustrated the movement of proanthocyanidins precursors into the vacuole (Terrier et al. 2009). Tannins are accumulated in specific vacuoles (“tannin

vacuoles") and act as deterrents to herbivores and fungi. Beyond astringency, tannins also confer bitterness, which is due to the lowest molecular weight tannins.

The predominant grape berry pigments are the anthocyanins, exclusively produced by red varieties. Besides anthocyanins,carotenoids, xanthophylls, and flavonols, such as quercetin, are present in both red and white varieties, but they are more important in the colour determination of white grapes (Jackson 2008). Pigments are generally confined to the vacuoles of a few cell layers immediately below the epidermis. A few cultivars, called "teinturiers", such as Alicante Bouschet, also contain anthocyanins in the mesocarp cells. Whereas flavonoid pigments are deposited in cell vacuoles, carotenoids accumulate predominantly in plastids (Jackson 2008).

Water-soluble anthocyanins are synthesised at the cytosolic surface of the endoplasmic reticulum and further transported to the vacuole, where they are usually sequestered, after being glycosylated (Grotewold 2004). Also, some enzymes involved in anthocyanin biosynthesis may be tonoplast-bound (Fritsch and Griesbach 1975). Spherical pigmented inclusions are present in the vacuoles of grape cells (Conn et al. 2003, 2010). Sequestration of anthocyanins by anthocyanic vacuolar inclusions (AVIs), loosely termed anthocyanoplasts, is believed to increase their stability and to reduce inhibition of certain vacuolar enzymes (Conn et al. 2003, 2010). Anthocyanoplasts start as vesicles in the cytosol and appear membrane-bound (Pecket and Small 1980, Nozzolillo and Ishikura 1988). Once in the vacuole, many factors influence the *in vivo* pigmentation provided by anthocyanins (Niloufer and Grotewold 2005). As anthocyanin synthesis and accumulation proceed, the anthocyanin content of the outer hypodermal layer(s) approaches saturation. At this time, anthocyanin combines in self-association or co-pigment complexes (Jackson 2008). In some cultivars, a decline in anthocyanin content is observed near or after maturity, probably due to β-glycosidases and peroxidases activities (Jackson 2008).

Anthocyanins assume their distinct colour after being transported to the vacuole and this compartmentation also decreases the feedback inhibition of cytosolic biosynthetic enzymes. The presence and number of hydroxyl groups, methylation and sugar moiety produce red, violet and blue coloration. Anthocyanins are red when acidic, colourless at pH 4.0, and purple above pH 4.5. Under alkaline conditions, a blue colour can be produced. The causes of blue colour in some cultivars are not known. The blue colour of other plant tissues has been attributed to anthocyanin complexes with alkaline metals or to co-pigmentation in anthocyanin-flavonoid complexes (Mullins et al. 1992).

The expression of the genes involved in anthocyanin biosynthesis is induced by light in seedlings and cell cultures, but the effect of light on the transcriptional activity of the anthocyanin pathway in berry skins is yet to be determined (reviewed by Boss and Davies 2009). Interestingly, light induces an alteration of anthocyanins distribution within vacuolar compartments (Niloufer and Grotewold 2005). Moreover, the temperature influences anthocyanin accumulation in grape berries with higher temperatures generally decreasing total anthocyanin levels (Boss and Davies 2009).

While the biosynthesis and regulation of anthocyanins has been extensively described, little is known on their sequestration in the vacuole and to what extent their colour is affected by storage (Niloufer and Grotewold 2005). Recently, and thanks to the sequencing of the grapevine genome (Jaillon et al. 2007), two grapevine proteins belonging to the Multidrug and Toxic Extrusion (MATE) family, anthoMATE1 (AM1) and anthoMATE3 (AM3), have been implicated in the mediated transport of specifically acylated anthocyanin (Gomez et al. 2009). Vacuolar sequestration of anthocyanins is an important process for cell survival because anthocyanins are believed to be toxic (reviewed by Boss and Davies 2009).

Vacuolar compartmentation of aroma compounds

Several hundred volatile compounds have been identified in ripe grapes. Aroma compounds are mostly accumulated in the exocarp (skin) tissue, as already pointed out. However, some volatile compounds accumulate differentially between the exocarp and the mesocarp (Luan and Wüst 2002). The final mixture of secondary metabolites in ripe grapes depends on multiple variables, including the grape variety used, the environmental conditions during the growing season, and management of the vineyard and harvest date (reviewed by Dunlevy et al. 2009). The major groups of aroma and flavour compounds produced in grapes are terpenoids, norisoprenoids (mainly C13 norisoprenoids), aromatics and aliphatics, and also organo-sulphur compounds (with a thiol function) and methoxypyrazines (Table 2).

Most of the literature on grape berry aroma compounds does not specify the vacuole as the main storage compartment for such compounds. Instead, it has been speculated that, after *véraison*, the plastids that lose their chlorophyll are the site of terpenoid and norisoprenoid synthesis and storage, but in other plants such as in *Pinus* species terpenoids synthesis is carried out by the endoplasmic reticulum (De 2000). Nevertheless, these secondary metabolites are frequently accumulated as glycosides (De 2000, Terrier et al. 2009, Dunlevy et al. 2009), and a number of glycosides also exist in vacuolar sap. Besides their general storage function, the vacuoles are also involved in the biosynthesis of higher saccharides from mono- or disaccharides and the site of glycosylation and production of various metabolites. Thus, given that glycosylation increases solubility and mobility and facilitates transport and storage processes, the vacuole may act as a reservoir of the glycosylated aroma compounds. Indeed, more recently, Lund and Bohlmann (2006) reported that aromas such as terpenes, norisoprenoids and thiols stored as sugar or amino acid conjugates are accumulated in the vacuoles of exocarp cells. Aromas such as terpenes, norisoprenoids and thiols conjugated with sugars or amino acids are accumulated in the vacuoles of exocarp cells (Lund and Bohlmann 2006).. In addition, Peyrot des Gachons and co-workers (2002) showed that the S-cysteine

conjugates are accumulated in the vacuoles where the glutathione moiety is cleaved by a peptidase, which yields a specific cysteine conjugate.

Many scientific advances have been achieved in understanding how the tonoplast machinery promotes the passage and accumulates in the vacuole lumen such a variety of compounds like ions, water, sugars, organic acids, phenolics, aromas, alkaloids, enzyme inhibitors and toxins, and exclude others, but this area of research is still wide open.

4. Conclusions and Prospects

The vacuole is a conspicuous organelle that plays a central role during grape berry development and ripening. Despite the importance and uniqueness of fruit vacuoles, especially grape berry vacuoles, we know little about vacuolar functions and vacuolar transporters. Proteomic methodologies, functional analysis and molecular characterization of tonoplast transporters should allow significant progress in our understanding of vacuole function (Maeshima 2001, Carter et al. 2004). Thus, the isolation and purification of intact vacuoles from grape cells are a prerequisite to understanding the physiology of this organelle. However, the mechanisms that control vacuole identity, as well as those controlling vacuole fusion or division are poorly unknown, although some information was already collected in yeasts (Baars et al. 2007). Also, the knowledge of how the tonoplast lipid composition - that for sure is changed during grape development and is influenced by environmental factors, like heat -, influences vacuole function and solute storage remains almost unexplored. The challenge for grape biologists is to deepen the study of vacuole structure, diversity, biochemistry and dynamics and to integrate this knowledge in the context of the cell/tissue type, physiology and developmental stage.

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Figures

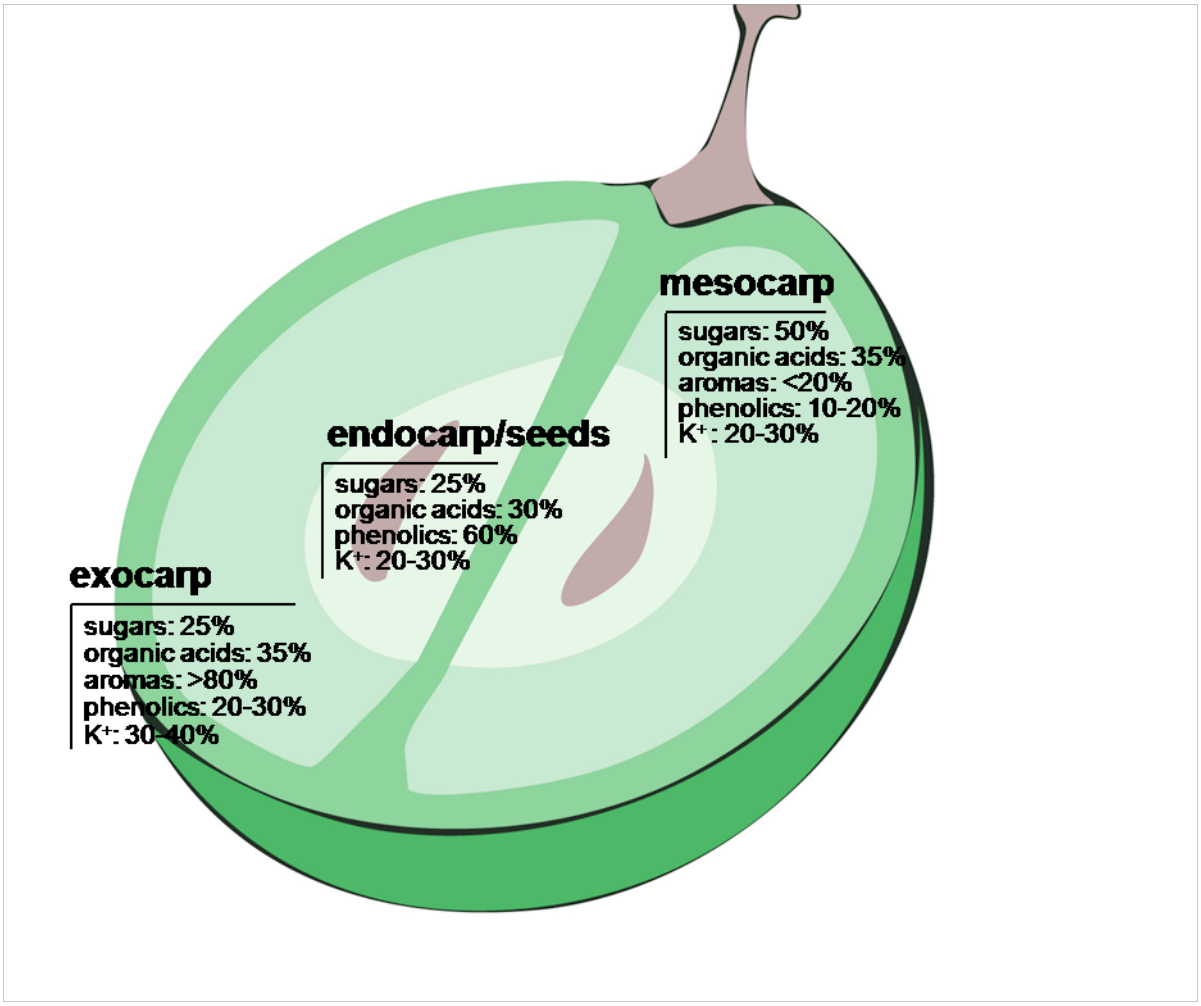

Figure 1. Structure of a ripe grape berry and pattern of solutes distribution (Coombe 1987, Conde et al. 2007, Jackson 2008). Figures indicate the percentage of each type of compound in a given compartment, relative to the whole berry.

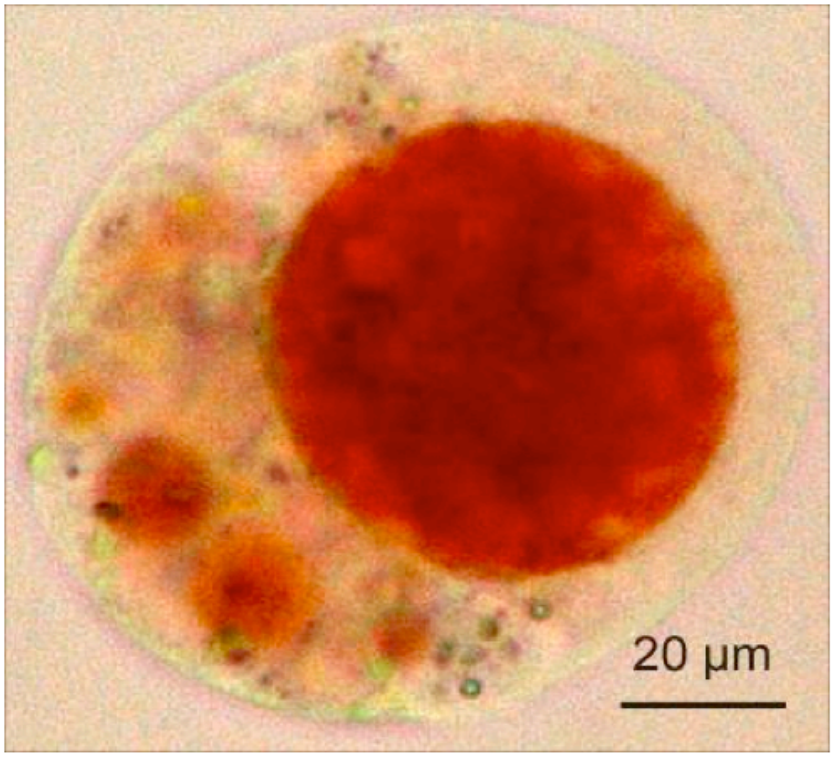

Figure 2. Protoplast from grape berry mesocarp labelled with Neutral Red to show the acidic nature and integrity of the vacuolar apparatus (Adapted from Fontes et al. 2010a).

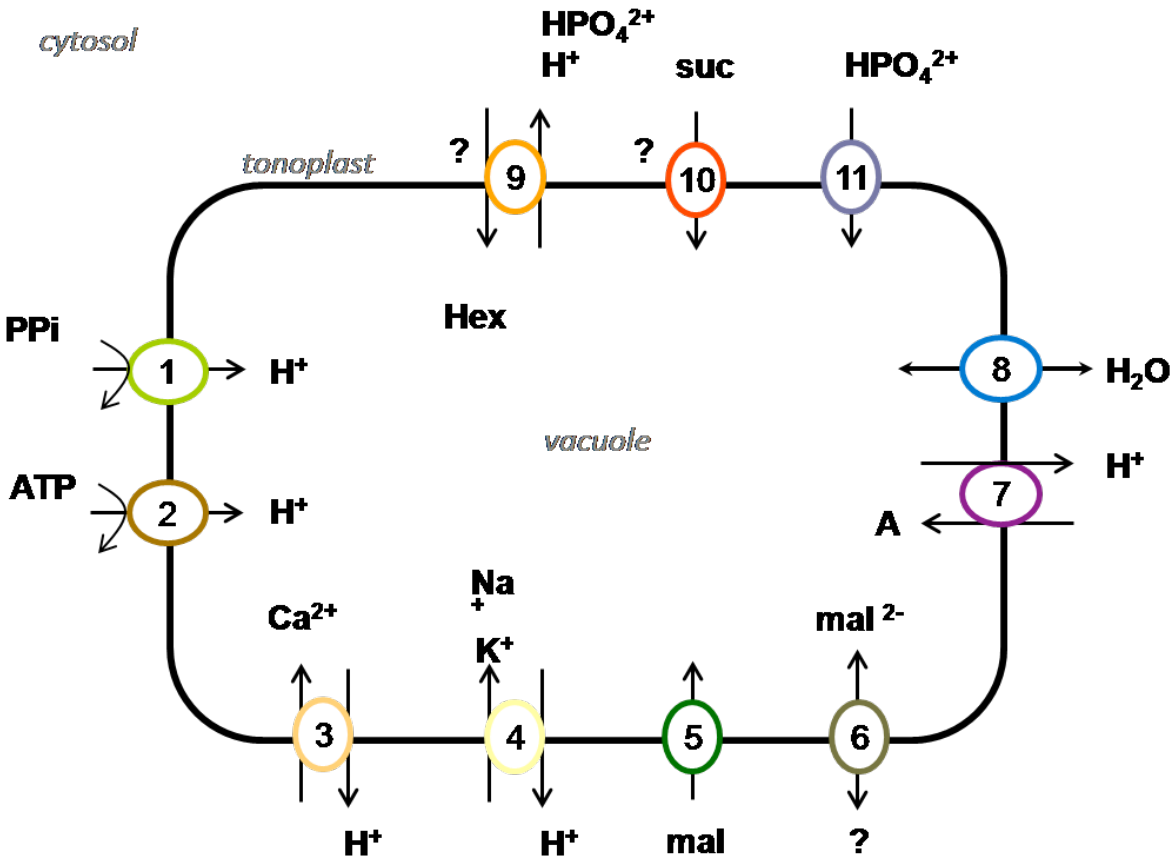

Figure 3. Grape berry vacuolar transport systems identified at molecular level or postulated from transport experiments. 1, V-PPase; 2, V-ATPase (Terrier et al. 1998; Fontes et al. 2010b); 3, Ca^{2+}/H^{+} antiport system (Fontes et al. 2010b); 4, cation/H^{+} antiporter (VvNHX; Hanana et al. 2007); 5, malate (mal) transporter (VvtDT; Rongala 2008); 6, malate channel (VvALMT9; Rongala 2008); 7, MATE transporter implicated in the uptake of acylated anthocyanin (A) (Gomez et al., 2009); 8, tonoplast intrinsic proteins (TIPs; Fouquet et al. 2008); 9, monosaccharide transporter; 10, sucrose (suc) transporters; 11, phosphate transporter (N. Fontes and co-workers, unpublished data, 2011). PPi, pyrophosphate.