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, color, texture, flavor, and pathogen susceptibility, mostly due to changes in vacuolar content. Most aroma and flavor 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 veraison, 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

33 electrochemical gradient across the tonoplast, which, in turn, energizes the uptake of charged and

34 uncharged solutes. Several tonoplast proteins mediating the uptake of sugars, organic acids,

35 water, ions and anthocyanins have been cloned and some of them have been functionally

36 characterized. The present review focuses on the storage function of vacuoles, as well as on their

37 structure and diversity in relation to development and ripening of the grape berry.

38 *Key words:* grape berry, vacuole, tonoplast transporters, sugars, organic acids, phenolics, aroma

39 compounds

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41 *1. Introduction*

42 Grape (*Vitis vinifera* L.) is a major crop worldwide. The majority of fruit production is

43 processed into wine, but significant portions are consumed fresh, dried into raisins, processed

44 into non-alcoholic juice, and distilled into spirits (reviewed by Conde et al. 2007). Berry content

45 at harvest is a major parameter for wine quality. The size and the quality of the berries mainly

46 depend on water, sugars (glucose and fructose), organic acids (malic and tartaric acids), phenolic

47 compounds (anthocyanidins and tannins) and aroma precursors.

48 The vacuoles are an essential organelle for plant cell physiology and therefore for plant life

49 (Martinoia et al. 2007). They are responsible for the high cell surface-to-protoplasmic volume

50 ratio required for extensive exchanges of material and information between plant cells and their

51 environment. The vacuole is surrounded by a biological membrane called the tonoplast which

52 separates the vacuolar lumen from the cytosol. Phospholipids, sterols, and ceramide

53 monohexoside(s) are the major lipid classes in the tonoplast and plasma membrane, but the

54 content of phospholipids on a protein basis is higher in the tonoplast (Yoshida and Uemura,

55 1986). Together with the cell wall, they control turgor, which is basic to cell hydraulic stiffness

56   and plant growth (Marty 1999). Recognized functions of the vacuole also encompass storage

57   (ions, metabolites, and proteins), digestion, pH and ion homeostasis, biotic and abiotic defence

58   responses, toxic compound sequestration, and pigmentation (Marty 1999, Martinoia et al. 2000,

59   Carter et al. 2004). The volume of the grape berry cells is largely occupied by a central vacuole.

60   Besides its multifaceted roles, the vacuole of grape berry cells has attracted attention mainly

61   because the storage function contributes directly to the quality of the fruit. Vacuoles are the main

62   reservoir of grape berry cells for sugars, organic acids, aromas, flavours, ions and water, that are

63   differently distributed throughout berry tissues (Figure 1).

64   Many scientific advances have led to an increased understanding of physiological,

65   biochemical, and molecular aspects of grape berry maturation; however, little is known about the

66   mechanisms, coordination, regulation and environmental sensitivity of the transport steps

67   involved in vacuolar accumulation of solutes (Shiratake and Martinoia 2007). More generally,

68   despite the importance and uniqueness of fruit vacuoles, the identity and functioning of vacuolar

69   transporters still need further investigation at the molecular level (Maeshima 2001, Shimaoka et

70   al. 2004, Carter et al. 2004, Endler et al. 2006).

71   The present review focuses on the diversity and biochemistry of the grape berry

72   components, which contribute to the organoleptic properties of the fruit and wine, in relation with

73   their vacuolar compartmentation during berry development and ripening. Emphasis will be given

74   to the storage function of the vacuoles, which rely on the coordinated activity of transport

75   proteins such as proton pumps and antiporters. Changes in the vacuolation degree, as well as

76   vacuole morphological diversity will be related to the physiological role of this highly dynamic

77   organelle during fruit development and ripening.

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79

80   ### *2. Structure and biochemical diversity of grape berry vacuoles*

81   Plant vacuoles are highly dynamic, multifunctional organelles which provide the primary

82   site of macromolecule storage and turnover. These organelles, that occupy as much as 90% of

83   most mature cells, are an integral part of the endomembrane system, serving as the terminal

84   products of the secretory pathway (Marty 1999). The space-filling function of the vacuole is

85   essential for cell growth, because cell enlargement is accompanied by expansion of the vacuole

86   rather than of the cytosol (Maeshima 2001). Ripe grape berry flesh cells are very heterogeneous,

87   showing evident vacuolar diversity both in size (ranging from 1 to 50 μm of diameter) and

88   content. Vacuoles in these cells vary from large and small colourless vacuoles to numerous small

89   acidic vacuoles distributed throughout the cytoplasm, with a large central-vacuole also being

90   observable in some cells (Figure 2). The diversity of vacuolar functions parallels their diversity in

91   morphology, biochemistry, and biogenesis (Marty 1999). As grape berries develop, the vacuolar

92   system displays modifications in vacuole number, size, and in composition. As referred to above,

93   fruit volumetric growth after veraison primarily results of vacuolar enlargement by incorporation

94   of water and solute.

95   The complexity of the vacuolar system offers a rich field of future work for plant biologists.

96   The vacuolar fraction from *Arabidopsis thaliana* shows the presence of many small vesicles

97   attached to the main vacuole, whose intravesicular environment is also acidic as shown by neutral

98   red staining (Shimaoka et al. 2004). Also, in specialized cells like fleshy cells of the grape berry,

99   the vacuolar volume and content may vary widely (N. Fontes and co-workers, unpublished data,

100   2011). This diverse vacuolar morphology probably reflects the multiple roles of the vacuole

101   system, as evidenced by many reports that emphasize the existence of different kinds of vacuoles

102   in plants (Paris et al. 1996, Marty 1999, Bethke and Jones 2000). As early as 1876, from his

103   observations on the anthocyanin vacuoles of *Drosera,* Charles Darwin documented that in a

104   given tissue the shape, number and volume of vacuoles in a cell may vary (De 2000). Indeed,

105   more than one kind of vacuole has been observed in cells undergoing maturation (Bethke and

106   Jones 2000), where some vacuoles primarily function as storage organelles and other as lytic

107   compartments (Paris et al. 1996, Marty 1999, Bethke and Jones 2000, Jiang et al. 2001, Martinoia

108   et al. 2007).

109   Apart from the lytic (LV) and protein storage vacuoles (PSV), widely distributed in plant

110   cells (Paris et al. 1996, Marty 1999, Jauh et al. 1999, Jiang et al. 2001, Reisen et al. 2005),

111   "tannin vacuole", "mucilage vacuole", "lipid vacuole" and "phenolic vacuole" have been

112   described (De 2000). Minorsky (2001) reported that the "phenolic vacuoles" were the so-called

113   "specialized vacuoles". The distinguishing features of phenolic vacuoles include their high

114   phenolic content, avidity for some basic dyes (eg. neutral red), unusually acidic interior, great sap

115   viscosity, and great refractivity (Minorsky 2001). The avidity of phenolic vacuoles for basic dyes

116   is due to dye precipitation by endogenous phenols. Furthermore, because tannins are, in general,

117   amorphous astringent substances which combine with ferric salts to produce blue, black or green

118   colour in sap, phenolic vacuoles have been named as "A" type or "full cell sap", in contrast to

119   "B" type or "empty cell sap", which do not contain phenolics (De 2000). Secondary metabolites

120   are synthesized, degraded and stored by a series of integrated processes controlled mainly by

121   membranes and by the different physico-chemical conditions present in the different cellular

122   compartments. Because most of these metabolites are toxic to the plant itself, their vacuolar

123   compartmentation may improve the efficiency of their production and avoid harmful effects in

124   the cells (Roytrakul and Verpoorte 2007).

125   When attempting to study the vacuolar system of grape mesocarp cells, a precise

126 identification mechanism of each compartment is needed. The tonoplast-intrinsic proteins (TIPs)

127 are the most abundant vacuolar transporters (reviewed by Gomes et al. 2009), and several TIP

128 genes, typically found in individual plant species, are differentially regulated, suggesting that

129 different TIPs may be utilized under specific conditions (Bethke and Jones 2000). Jauh and co-

130 workers (1999) showed that different kinds of vacuoles are labelled with different combinations

131 of TIPs. As a result, they have proposed TIPs as markers of vacuole function and developmental

132 stage (Jauh et al. 1999). However, it remains to be confirmed if these antibodies identify TIP

133 orthologs in other species.

134   The use of fluorescent probes may also be useful in characterizing vacuole functions.

135 However, vacuoles contain large amounts of anthocyanins and flavonols producing an intense

136 autofluorescence, the emission of which varies according to local pH and ionic conditions. This

137 may impair the utilization of fluorescent probes (Johnson 2006).

138   Many fine modifications in cell structure, especially those concerning the vacuolation

139 degree, as well as vacuole morphological diversity, may result of environmental constraint and

140 fruit maturation stage. At veraison, the large vacuole splits into smaller vacuoles generating a

141 complex internal membrane structure. Changes in the vacuolation degree of grape cells may be

142 involved in the maintenance of turgor pressure or in the shift to storage and digestion, as reported

143 for root apex cells of soybean (Klymchuk et al. 2003).

144   After veraison, the grape berry tissues, and thus the vacuolar content, change from full-

145 scale defence against bird, insect and fungus, to an appealing sugary tissue, with reduced malic

146 acid content. At this time, massive sugar accumulation makes the berry very attractive to birds

147 and mammals and allows seed dispersion (Hardie et al. 1996). Most of the aroma and flavour

148 compounds are not evenly distributed in the berry tissues, and their composition and

149   concentration vary along with development and maturation. Accordingly, the vacuolation degree

150   or type of vacuole may also depend on the berry tissue. Prior to ripening, tannins and polyphenols

151   accumulate in outer mesocarp cells that surround the tissues of the peripheral vascular network,

152   and later on during ripening they accumulate in greatest abundance in the exocarp cells. Besides

153   storing tannin compounds, the "tannin rich cells", with "tannin vacuoles", may protect the

154   vascular parenchyma cells against UV light. Also, these cells possess vacuoles acting as

155   intermediate storage sites for the fluxes of assimilates that, as in other tissues, probably exit the

156   phloem of the peripheral vascular network prior to their re-entry into the symplast of the pericarp

157   parenchyma (Hardie et al. 1996).

158

159   ### *3. The vacuole as a storage compartment*

160   Major concerns for grape growers are the control of the ripening time, berry size and

161   colour, acidity and the relative range of volatile and non-volatile aroma and flavour compounds.

162   Therefore, understanding how and when various components accumulate in the berry, and how

163   berry development and maturation responds to environmental stress factors, is of critical

164   importance to adjusting grape growing practices and thus modifying wine typology (reviewed by

165   Conde et al. 2007).

166   As already stated, grape berry vacuoles accumulate sugars, organic acids, aromas, flavours,

167   ions and water (Figure 1). Each of these compounds is transported across the tonoplast by a

168   specific transporter protein that may be an active pump, a carrier or a channel. Some tonoplast

169   transporter proteins have been identified and functionally characterized in grape cells (Figure 3),

170   but solute compartmentation in the vacuoles of grapevine cells is still poorly documented.

171

172   *Two proton pumps energize the vacuolar membrane*

173   Grape berry vacuoles maintain an acidic pH, ranging from pH 2.5 in the green stage to pH

174   3.5 during ripening. The maintenance of ion and proton concentration gradients across the

175   tonoplast membrane is essential for acid and sugar homeostasis in the berry (Hanana et al. 2007).

176   The low pH of the vacuole of fruit cells is the result of two processes: i) proton pumping across

177   the tonoplast, and ii) synthesis and accumulation of organic acids in the vacuolar sap (Shiratake

178   and Martinoia 2007). Two distinct primary proton pumps, the vacuolar ATPase (V-ATPase) and

179   the vacuolar inorganic pyrophosphatase (V-PPase), generate a proton electromotive force, which,

180   in turn, allows the secondary active transport of inorganic ions, sugars and organic acids

181   (Blumwald, 1987; Maeshima, 2001; Martinoia et al., 2007; Fontes et al. 2010b). The V-PPase is

182   generally more active than the V-ATPase in young tissues with relatively high amounts of

183   pyrophosphate (PPi) that is a by-product of biosynthetic pathways (Martinoia et al. 2007).

184   However, the V-PPase is the predominant vacuolar proton pump in tonoplast vesicles from

185   mature grape berries (Terrier et al., 1998) and intact vacuoles from CSB (Cabernet Sauvignon

186   Berry) suspension cultured cells (Fontes et al. 2010b). Intact vacuoles are good experimental

187   models to monitor the mechanisms of vacuolar acidification and solute uptake (Fontes et al.

188   2010b).

189

190   *Vacuolar compartmentation of sugars in grape berry*

191   Although the transport mechanisms of monosaccharides and disaccharides at the plasma

192   membrane level are reasonably understood in several plants, including grapevine, information on

193   tonoplast sugar transporters is still limited. However, some tonoplast monosaccharide

194   transporters (TMT) have been recently reported as mediating a proton-coupled antiport

195   mechanism. Three AtTMT (*Arabidopsis thaliana* tonoplast monosaccharide transporters)

196 isoforms were localized at the tonoplast by fusion with the green fluorescent protein (GFP)

197 (Neuhaus 2007) and the tonoplast glucose/H^+ antiporter AtVGT1 (At3g03090) was characterized

198 in the same plant model (Aluri and Büttner 2007). In *V. vinifera*, the hexose transporter VvHT6 is

199 presumed to be targeted to the tonoplast (reviewed by Agasse et al. 2009). The sequence of

200 VvHT6 is similar to that of the three AtTMT with an extended loop between the transmembrane

201 helixes six and seven (Büttner 2007; Hayes *et al.* 2007), and its pattern of expression is consistent

202 with a role in postveraison import of hexoses into the vacuole. Uptake activities of the plasma

203 membrane hexose transporters VvHT1, VvHT4 and VvHT5 have been demonstrated by

204 heterologous expression in the hxt-null mutant yeast, but attempts to confirm the transport

205 activity of VvHT6 has had little success (reviewed by Agasse et al. 2009).

206 Besides their role in sugar storage, vacuoles are also involved in the biosynthesis of higher

207 saccharides from mono- or disaccharides. Also, vacuoles are likely the site for glycosylation and

208 production of various metabolites (De 2000).

209

210 *Water incorporation in the grape berry and the role of aquaporins*

211 Vine water deficit has a clear implication in wine composition and sensory attributes (Roby

212 et al. 2004). It generally leads to smaller berries (Bravdo et al. 1985, Kennedy et al. 2002,

213 Matthews et al. 1990), thus increasing the skin to juice ratio, which, in turn, may increase the

214 concentration of anthocyanins and phenolics in the must and wine (Reviewed by Conde et al.

215 2007).

216 Most of the berry volume gained before veraison is due to water import through the xylem,

217 whereas most of the post-veraison gain is due to water import through the phloem. This strong

218 phloem component at veraison might explain the insensitivity of the berry to plant water deficits

219 (Matthews et al. 1987). In addition, the shift of phloem unloading from symplastic to apoplastic

220 pathway at veraison is associated with sugar accumulation at high levels in sink organs (Patrick

221 1997, Zhang et al. 2006), favouring the maintenance of a turgor pressure gradient. Moreover, the

222 involvement of cell compartmentation of water and solutes makes more and more difficult for the

223 leaves to extract water from ripening berries (Keller et al. 2006). The co-expression of some

224 aquaporins and sugars transporters suggests a functional link between sugar and water fluxes

225 during the processes of unloading and sugar accumulation in the vacuoles of the flesh cells

226 (Delrot et al. 2001). Aquaporins are specialized proteins in the major intrinsic proteins (MIP)

227 family that are implicated in water transport across biological membranes (Fouquet et al. 2008,

228 Gomes et al. 2009).

229 Eight cDNAs encoding putative *Vitis* aquaporins (PIPs, Plasma Membrane Intrinsic Proteins and

230 TIPs, Tonoplast Intrinsic Proteins) were found to be mostly expressed in roots, eventually

231 enhancing and regulating water permeability (Baiges et al., 2001). The aquaporin VvPIP1A

232 mediates water transport and is mainly expressed in the berries after veraison (Picaud et al.,

233 2003). Moreover, after the release of the grapevine genome in 2007 (Jaillon et al., 2007), Fouquet

234 and co-workers (2008) have identified 28 genes encoding putative aquaporins, and they have

235 isolated 9 cDNAs encoding putative PIP and TIP aquaporins from grape berries at various

236 developmental stages. Aquaporin gene expression is strongly regulated during berry development

237 and globally decreases during ripening. The tonoplast aquaporin VvTIP2;1 and the plasma

238 membrane aquaporin VvPIP2;1 are highly expressed in dividing and elongating cells and in cells

239 involved in water and solutes transport (Fouquet et al. 2008). More recently, TIP2;1 was

240 confirmed to transport water when individually expressed in *Xenopus* oocytes (Vandeleur et al.,

241 2009).

242   *Vacuolar compartmentation of malic and tartaric acids*

243   The final organic acid content of the berries depends on the amount of acid synthesized,

244   stored in the vacuole and degraded during the ripening stages. Organic acids are produced both in

245   the leaves and fruits, but their biosynthetic mechanisms and compartmentation in the grape berry

246   cells remain poorly understood. Along with berry development and ripening, the berry acid

247   content varies. As reported above malic acid rapidly accumulates at early stages and decrease at

248   the onset of ripening. Tartaric acid amount is kept constant and its concentration declines mainly

249   due to dilution as berry volume increases.

250   During the vegetative growth phase, the sugars coming from photosynthesis in the leaves

251   are transformed into malic acid which accumulates in the vacuoles of pericarp cells (Schulze et

252   al. 2002). Also, it is believed that green berries are photosynthetically active and produce malic

253   acid as a source of carbon and energy (Sweetman et al. 2009). Contrarily to many other fruits,

254   grapes are incapable of storing significant amounts of starch. Malic acid is accumulated in the

255   fleshy cells at the end of the berry's first growth phase and reaches a maximal value just prior to

256   veraison. At veraison, due to the severe inhibition of the glycolytic pathway, malic acid import

257   from the large central vacuole allows energy production, lowering grape malate levels. The

258   decrease in malic acid in the grape berry at the onset of ripening also results from reduced malate

259   synthesis, but the reduction in the amount of acid translocated from the leaves to the berries may

260   also play a significant role.

261   Malate is accumulated in vacuoles at very high concentrations (> 300 mM) and the acid

262   exchange across the tonoplast is believed to be driven by the electrochemical membrane potential

263   difference (Martinoia et al. 2007). In Arabidopsis, malate exchange between the vacuole and the

264   cytoplasm is mediated by AttDT, a tonoplast malate transporter (Emmerlich et al. 2003) and by

265   AtALMT9, a tonoplast malate channel (Kovermann et al. 2007). Hurth et al. (2005) reported that

266   the activity of AttDT is critical for pH homeostasis.

267   In grape berry, four good malate transporter candidates have been identified by blast

268   analysis of the *Vitis vinifera* genome with the AtALMT9 protein sequence (Rongala 2008). These

269   genes are developmentally regulated. VvALMT9:1 and VvALMT9:2 showed post-*veraison*

270   expression, while VvALMT9:3 is expressed at high levels before veraison, and VvALMT9:4 is

271   poorly expressed. A single *AttDT* homologue has been identified in *V. vinifera* (Rongala 2008).

272   An homologue of *AttDT* has been implicated in citrate efflux in citrus fruits (Shimada et al.

273   2006).

274   As already stated, the drop in tartaric acid content, from 150 mM at *veraison* to 25-75 mM

275   at maturity, is mostly due to the increase of the berry size. Also, the free versus salt state of

276   tartaric acid generally changes throughout maturation, contrarily to malic acid that generally

277   remains as a free acid. Little is known about the biochemical mechanisms involved in tartaric

278   acid accumulation in the vacuole of grape cells.

279

280   *Vacuolar compartmentation of potassium*

281   Several ions, such as potassium, calcium, magnesium and chloride are implicated in

282   numerous physiological processes that impact fruit quality and, ultimately, wine taste and

283   flavour. Therefore, the study of the biochemical steps involved in their compartmentation and

284   interaction with other solutes is of major importance for plant physiologists and viticulturists.

285   Potassium is an essential macronutrient for grape berry growth and development, and a

286   well-known enzyme activator. It is the main cation in must and wine (~ 900 mg/L; reviewed by

287   Conde et al. 2007). Potassium is the main osmoticum in skin cells, as sugar is in the flesh.

288   Because potassium may accumulate in the vacuole, it affects several transport processes across

289 the tonoplast membrane. Thus, potassium uptake may increase the release of malic acid,

290 favouring its metabolism in the cytoplasm (Jackson 2008, Davies et al. 2006).

291 Recently, two cDNAs encoding potassium plasma membrane transporters (VvKUP1 and

292 VvKUP2) from grape berries were isolated and their function was demonstrated by heterologous

293 expression in an *Escherichia coli* mutant deficient in potassium uptake (Davies et al. 2006). The

294 two transporters are highly expressed in the berry skin during the first phase of berry

295 development, suggesting that, at this time, they play a role either in potassium uptake into the

296 berries or in its compartmentation into the skin cells. However the transcript levels of both

297 transporters are still significant at post-*veraison* suggesting that VvKUP1 and VvKUP2 may

298 therefore continue to contribute to potassium homeostasis throughout berry repening. At the

299 tonoplast level, a NHX-type cation/H^+ antiporter was recently cloned and functionally

300 characterized (Hanana et al. 2007). VvNHX1 couples the passive movement of H^+ out of the

301 vacuole to the active incorporation of monovalent cations (mainly K^+ and Na^+), playing an

302 important role in vacuolar ion homeostasis in grape berries.

303

304 *Vacuolar compartmentation of phenolic compounds*

305 Phenolic compounds are an extended family of secondary metabolites in grape berries

306 (Table 1). They are involved in plant protection because they are active growth inhibitors of other

307 living systems. Also, they add colour and flavour to the fruit, contributing to the mouthfeel,

308 quality and palatability of red wines. The major flavours associated with polyphenols are

309 bitterness and astringency. Additionally, they may play important beneficial roles for human

310 health as strong anti-oxidants (reviewed by Conde et al. 2007) and as activators of the human

311 oestrogen receptor alpha (Chalopin et al. 2010). Many secondary metabolites, particularly

312 phenolic compounds are frequently accumulated as glycosides, which increases their solubility,

313 transport and storage ability (De 2000). Non-flavonoid phenolics accumulate primarily in the

314 vacuoles of mesocarp cells, but flavonoids accumulate in the dermal cells of the skin tissue

315 (Table 1).

316   Tannins or proanthocyanidins are polymers of flavan-3-ols and are the most abundant class

317 of soluble polyphenolics in grape berries. A number of studies involving the identification of

318 several enzymes, transcriptional regulators of proanthocyanidins biosynthesis and transporters

319 have illustrated the movement of proanthocyanidins precursors into the vacuole (Terrier et al.

320 2009). Tannins are accumulated in specific vacuoles ("tannin vacuoles") and act as deterrents to

321 herbivores and fungi. Beyond astringency, tannins also confer bitterness, which is due to the

322 lowest molecular weight tannins.

323   The predominant grape berry pigments are the anthocyanins, exclusively produced by red

324 varieties. Besides anthocyanins,carotenoids, xanthophylls, and flavonols, such as quercetin, are

325 present in both red and white varieties, but they are more important in the colour determination of

326 white grapes (Jackson 2008). Pigments are generally confined to the vacuoles of a few cell layers

327 immediately below the epidermis. A few cultivars, called "teinturiers", such as Alicante

328 Bouschet, also contain anthocyanins in the mesocarp cells. Whereas flavonoid pigments are

329 deposited in cell vacuoles, carotenoids accumulate predominantly in plastids (Jackson 2008).

330   Water-soluble anthocyanins are synthesised at the cytosolic surface of the endoplasmic

331 reticulum and further transported to the vacuole, where they are usually sequestered, after being

332 glycosylated (Grotewold 2004). Also, some enzymes involved in anthocyanin biosynthesis may

333 be tonoplast-bound (Fritsch and Griesbach 1975). Spherical pigmented inclusions are present in

334 the vacuoles of grape cells (Conn et al. 2003, 2010). Sequestration of anthocyanins by

335 anthocyanic vacuolar inclusions (AVIs), loosely termed anthocyanoplasts, is believed to increase

336   their stability and to reduce inhibition of certain vacuolar enzymes (Conn et al. 2003, 2010).

337   Anthocyanoplasts start as vesicles in the cytosol and appear membrane-bound (Pecket and Small

338   1980, Nozzolillo and Ishikura 1988). Once in the vacuole, many factors influence the *in vivo*

339   pigmentation provided by anthocyanins (Niloufer and Grotewold 2005). As anthocyanin

340   synthesis and accumulation proceed, the anthocyanin content of the outer hypodermal layer(s)

341   approaches saturation. At this time, anthocyanin combines in self-association or co-pigment

342   complexes (Jackson 2008). In some cultivars, a decline in anthocyanin content is observed near

343   or after maturity, probably due to β-glycosidases and peroxidases activities (Jackson 2008).

344   Anthocyanins assume their distinct colour after being transported to the vacuole and this

345   compartmentation also decreases the feedback inhibition of cytosolic biosynthetic enzymes. The

346   presence and number of hydroxyl groups, methylation and sugar moiety produce red, violet and

347   blue coloration. Anthocyanins are red when acidic, colourless at pH 4.0, and purple above pH

348   4.5. Under alkaline conditions, a blue colour can be produced. The causes of blue colour in some

349   cultivars are not known. The blue colour of other plant tissues has been attributed to anthocyanin

350   complexes with alkaline metals or to co-pigmentation in anthocyanin-flavonoid complexes

351   (Mullins et al. 1992).

352   The expression of the genes involved in anthocyanin biosynthesis is induced by light in

353   seedlings and cell cultures, but the effect of light on the transcriptional activity of the anthocyanin

354   pathway in berry skins is yet to be determined (reviewed by Boss and Davies 2009).

355   Interestingly, light induces an alteration of anthocyanins distribution within vacuolar

356   compartments (Niloufer and Grotewold 2005). Moreover, the temperature influences anthocyanin

357   accumulation in grape berries with higher temperatures generally decreasing total anthocyanin

358   levels (Boss and Davies 2009).

359   While the biosynthesis and regulation of anthocyanins has been extensively described, little

360   is known on their sequestration in the vacuole and to what extent their colour is affected by

361   storage (Niloufer and Grotewold 2005). Recently, and thanks to the sequencing of the grapevine

362   genome (Jaillon et al. 2007), two grapevine proteins belonging to the Multidrug and Toxic

363   Extrusion (MATE) family, anthoMATE1 (AM1) and anthoMATE3 (AM3), have been implicated

364   in the mediated transport of specifically acylated anthocyanin (Gomez et al. 2009). Vacuolar

365   sequestration of anthocyanins is an important process for cell survival because anthocyanins are

366   believed to be toxic (reviewed by Boss and Davies 2009).

367

368   *Vacuolar compartmentation of aroma compounds*

369   Several hundred volatile compounds have been identified in ripe grapes. Aroma compounds

370   are mostly accumulated in the exocarp (skin) tissue, as already pointed out. However, some

371   volatile compounds accumulate differentially between the exocarp and the mesocarp (Luan and

372   Wüst 2002). The final mixture of secondary metabolites in ripe grapes depends on multiple

373   variables, including the grape variety used, the environmental conditions during the growing

374   season, and management of the vineyard and harvest date (reviewed by Dunlevy et al. 2009). The

375   major groups of aroma and flavour compounds produced in grapes are terpenoids, norisoprenoids

376   (mainly C13 norisoprenoids), aromatics and aliphatics, and also organo-sulphur compounds (with

377   a thiol function) and methoxypyrazines (Table 2).

378   Most of the literature on grape berry aroma compounds does not specify the vacuole as the

379   main storage compartment for such compounds. Instead, it has been speculated that, after

380   *veraison*, the plastids that lose their chlorophyll are the site of terpenoid and norisoprenoid

381   synthesis and storage, but in other plants such as in *Pinus* species terpenoids synthesis is carried

382   out by the endoplasmic reticulum (De 2000). Nevertheless, these secondary metabolites are

383   frequently accumulated as glycosides (De 2000, Terrier et al. 2009, Dunlevy et al. 2009), and a

384   number of glycosides also exist in vacuolar sap. Besides their general storage function, the

385   vacuoles are also involved in the biosynthesis of higher saccharides from mono- or disaccharides

386   and the site of glycosylation and production of various metabolites. Thus, given that

387   glycosylation increases solubility and mobility and facilitates transport and storage processes, the

388   vacuole may act as a reservoir of the glycosylated aroma compounds. Indeed, more recently,

389   Lund and Bohlmann (2006) reported that aromas such as terpenes, norisoprenoids and thiols

390   stored as sugar or amino acid conjugates are accumulated in the vacuoles of exocarp cells.

391   Aromas such as terpenes, norisoprenoids and thiols conjugated with sugars or amino acids are

392   accumulated in the vacuoles of exocarp cells (Lund and Bohlmann 2006). In addition, Peyrot des

393   Gachons and co-workers (2002) showed that the S-cysteine conjugates are accumulated in the

394   vacuoles where the glutathione moiety is cleaved by a peptidase, which yields a specific cysteine

395   conjugate.

396   Many scientific advances have been achieved in understanding how the tonoplast

397   machinery promotes the passage and accumulates in the vacuole lumen such a variety of

398   compounds like ions, water, sugars, organic acids, phenolics, aromas, alkaloids, enzyme

399   inhibitors and toxins, and exclude others, but this area of research is still wide open.

400

401   *4. Conclusions and Prospects*

402   The vacuole is a conspicuous organelle that plays a central role during grape berry

403   development and ripening. Despite the importance and uniqueness of fruit vacuoles, especially

404   grape berry vacuoles, we know little about vacuolar functions and vacuolar transporters.

405 Proteomic methodologies, functional analysis and molecular characterization of tonoplast

406 transporters should allow significant progress in our understanding of vacuole function

407 (Maeshima 2001, Carter et al. 2004). Thus, the isolation and purification of intact vacuoles from

408 grape cells are a prerequisite to understanding the physiology of this organelle. However, the

409 mechanisms that control vacuole identity, as well as those controlling vacuole fusion or division

410 are poorly unknown, although some information was already collected in yeasts (Baars et al.

411 2007). Also, the knowledge of how the tonoplast lipid composition—which is changed during

412 grape development and is influenced by environmental factors such as heat—influences vacuole

413 function and solute storage remains almost unexplored. The challenge for grape biologists is to

414 deepen the study of vacuole structure, diversity, biochemistry and dynamics and to integrate this

415 knowledge in the context of the cell/tissue type, physiology and developmental stage.

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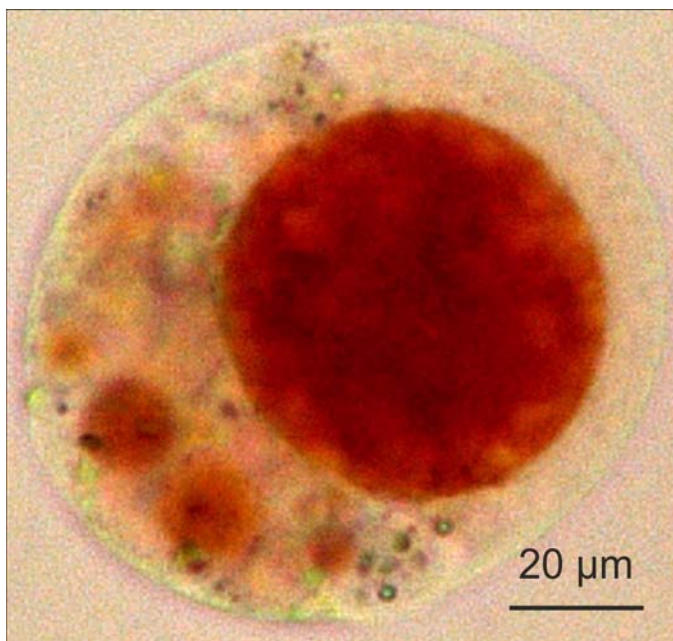729   involved in developmental onset of ripening in grape berry. Plant Physiol. 142:220-232.

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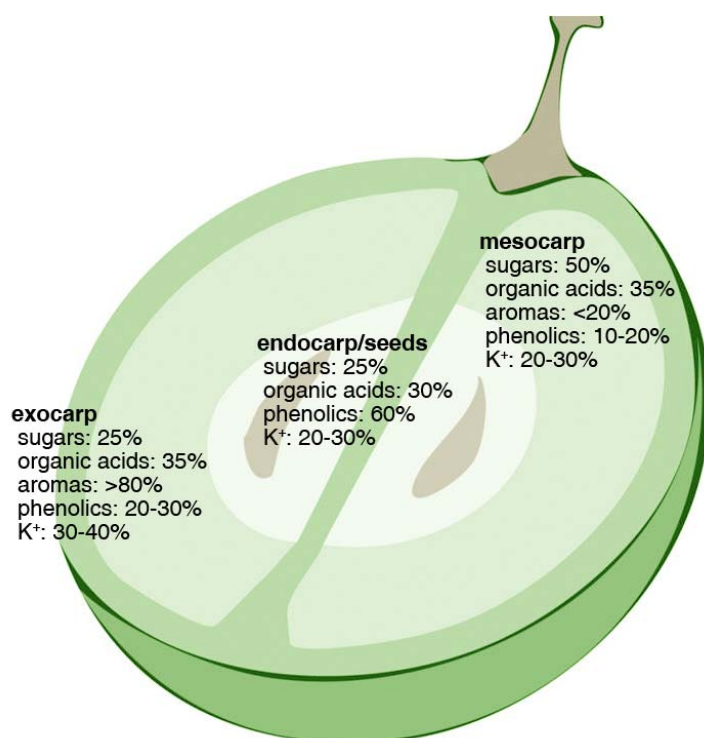
Figure 1. Structure of a ripe grape berry and pattern of solutes distribution (Coombe 1987, Conde et al. 2007, Jackson 2008). Figures indicate the percentage of each type of compound in a given compartment, relative to the whole berry.
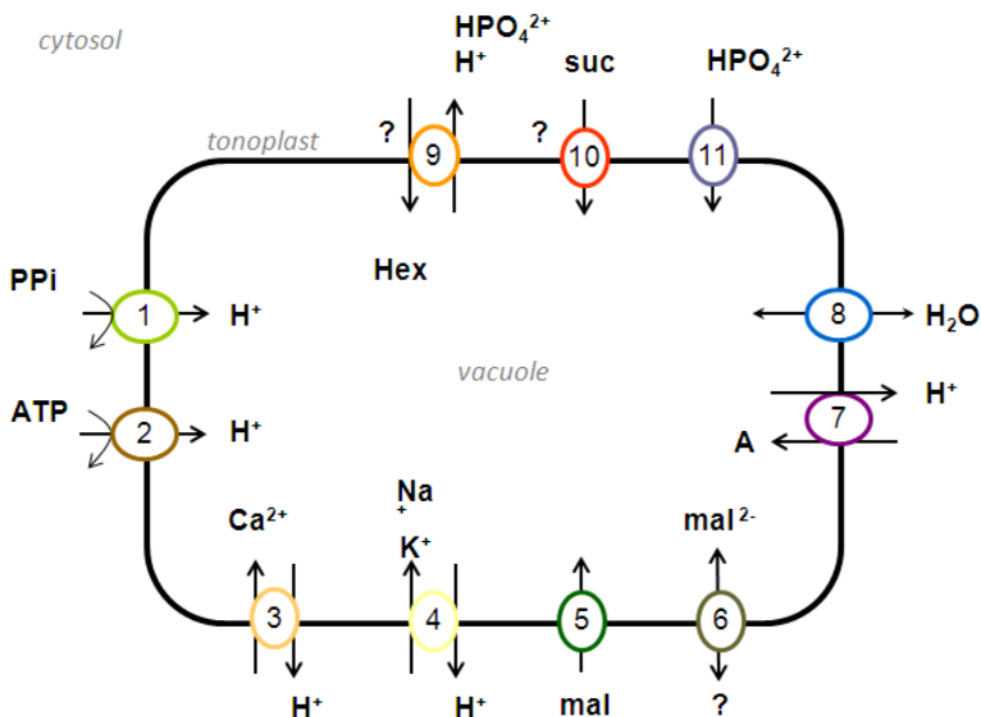
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Figure 2. Protoplast from grape berry mesocarp labelled with Neutral Red to show the acidic
nature and integrity of the vacuolar apparatus (Adapted from Fontes et al. 2010a).

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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.

Table 1. Phenolic compounds in the grape berry

Compound	Location			Biological role	Observations	References
	Skin	Flesh	Seeds			
Flavan-3-ols (flavonoid)	+++	-	+	Plant defence; Flavor: astringency and bitterness; Colour stability (wine)	Larger flavonoid group; Catechins (skin and seeds); gallocatechins (skin); Synthesis starts at early stages of development until *véraison*	Terrier et al. 2009 Lacopini et al. 2008
	+++	+	+	Flavor: astringency, and bitterness ; UV protection	Form cross-links between proteins and other molecules; Skin contains 89% of berry tannins and seeds 11%; Flesh contains only soluble tannins at low levels; Synthesis and accumulation starts at early stages of berry development	Kennedy 2008
Anthocyanins (flavonoid)	+++	+	-	Color; UV protection; anti-oxidant activity	Present in skin dermal cells of red varieties; Accumulated as glycosides; Malvidin: grape predominant glycoside; Flesh anthocyanins occurs in teinturier varieties; Anthocyanin vacuolar inclusions (AVI) – vacuolar structures of anthocyanins storage; MATE transporter mediates the incorporation of acylated anthocyanin	Boss and Davies 2009 Jackson 2008 Conn et al. 2003, 2010 Gomez et al. 2009
Non-flavonoid	-	++	+++	Plant defence	Includes: caftaric and coumaric acids, benzoic and cinnamic acids, among others; Non-flavonoid compounds and tannins are the most accumulated compounds in grape seeds; Synthesis is maintained throughout ripening.	Kramling and Singleton 1969 De 2000 Montealegre et al. 2006

Table 2. Aroma compounds in the grape berry

Group	Common compounds	Odor description	Aromatic precursor (odorless)	Location		Observations	References
				Skin	Flesh		
Terpenoids (Monoterpenes; Sesquiterpenes)	Monoterpenols: Linalol Geraniol Nerol	*rose* *rose* *rose*	Glycoside conjugates	+++	+	Mostly present in Muscat varieties as both free (volatile) and bound (non-volatile) forms; Nerol and geraniol concentrate in the skin; Both free and bound fractions tend to decline at maturity; Synthesised at ER and accumulated in the vacuoles	Gunata et al. 1985 Park et al. 1991
Norisoprenoids (C$_{13}$ norisoprenoids)	β-damascenone β-inonone TDN Vitispirane	*honey* *violet* *kerosene* *spicy, woody*	Glycoside conjugates	+	+++	Mostly accumulated as bound non-volatile compounds; only trace amounts of damascenone occurring as free volatile molecules; Products of carotenoid degradation; Levels increase after *véraison*; Accumulation in the berry not closely related to sugar concentration;	Lewinsohn et al. 2005 Razungles et al. 1993
Organo-sulfur compounds (Thiols)	3-sulfanylhexan-1-ol; 2-methylfuran-3-thiol; 4-methyl-4-sulphanylpentan-2-one; 3-sulfanylbutan-1-ol; 3-sulfanylpentan-1-ol;	*Sulphur, passion fruit, cat urine, box tree, rhurbarb;* *meaty* *box tree* *onion, leek*	S-cysteine conjugates	+	+	S-cystein conjugates largely restricted to the skin; Suffer further cleavage in the vacuole resulting in a specific cystein conjugate: Precursors appear differently during maturation	Tominaga et al. 1998 Mestres et al. 2000 Francis and Newton 2005
Methoxypyrazynes (Pyrazynes)	IPMP IBMP SBMP	*grapefruit* *asparagus, green pea* *green pepper* *beet, earthy*		+	-	Positive and negative contribution to wine aroma, flavour and mouthfeel; Vegetative character of Cabernet Sauvignon variety; Accumulation peak prior to *véraison* and further drop as the berry ripens	Belancic and Agosin 2007 Parr et al. 2007

TDN – 1,1,6-trimethyl-1,2-dihydronaphthalene; TPB – (E)-1-(2,3,6-trimethylphenyl)buta-1,3-diene; IPMP – isopropyl methoxypyrazine; IBMP – isobutyl methoxypyrazine; SBMP – sec-butyl methoxypyrazine; ER – Endoplasmic Reticulum