

# The Vacuolar Localization of Grapevine Peroxidase Isoenzymes Capable of Oxidizing 4-Hydroxystilbenes

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A study of the subcellular localization of peroxidase isoenzymes capable of oxidizing 4-hydroxystilbenes to viniferin-type compounds has been carried out in cultured cells derived from *Vitis vinifera* cv. Gamay berries. The study revealed that these isoenzymes are apparently located, in soluble form, in the vacuolar sap and are strongly inhibited by vacuolar anthocyani(di)ns in the concentration range found *in planta*. These results suggest that the inhibition of the 4-HS peroxidase system in vacuoles, as a consequence of anthocyani(di)n accumulation, could be responsible for the loss of viniferin production potential which accompanies grape berry veraison.

## Introduction

The accumulation of phytoalexin in plants in response to infection by pathogenic fungi is believed to be an important aspect of plant disease resistance. Thus, phytoalexins are utilized by plants to stop the growth of the attacking fungus [1]. In Vitaceae, the predominant phytoalexins have been described as derivatives of the 4-hydroxystilbene backbone [2] and so-called viniferins. The ability of several grape-vine varieties to produce these compounds has been used in the development of methods for screening grape-vines for resistance to infection by fungi [3].

The synthesis of viniferins takes place through a set of enzymes which first synthesize the 4-hydroxystilbene backbone (*i.e.* resveratrol, 3,5,4'-trihydroxystilbene) from phenylpropanoid precursors and malonyl-CoA [4] and then oxidize two or three 4-hydroxystilbene moieties to give viniferins

[5]. In this last oxidative coupling reaction, peroxidase (EC 1.11.1.7) is involved [5].

Although there are several studies on peroxidase localization in plant cells [6, 7], the high number of peroxidase isoenzymes in grape-vine cell homogenates [8] and the broad range of suitable electron donors for each peroxidase isoenzyme [9] make it very difficult to extrapolate the information to a specific substrate. Thus, to date, the subcellular localization of plant peroxidase isoenzymes capable of oxidizing 4-hydroxystilbenes (4-HS) remains an open question.

In this paper we report that 4-HS oxidizing peroxidase isoenzymes are mainly located in the vacuolar sap of Gamay grape-vine cell cultures. Subcellular fractionation and organelle preparation, in combination with kinetic and zymographic probes on the 4-HS oxidizing activity of grape-vine peroxidase isoenzymes, has permitted us to unravel the specific vacuolar localization and its control by anthocyanins, of the last step of the pathway which derives from phenylalanine metabolism, before merging with a 4-hydroxystilbene backbone, and leading to viniferins in Vitaceae.

## Materials and Methods

### Chemicals

Anthocyani(di)ns (cyanin chloride, cyanidin chloride, delphinidin chloride and peonidin chloride) were purchased from Extrasynthèse (Genay, France). 4-Hydroxystilbene (4-HS) was obtained

**Abbreviations:** 4-AAP, 4-aminoantipyrine; DEAE-dextran, diethylaminoethyl-dextran; Dextran-SO<sub>4</sub>, dextran-sulfate; EGTA, ethyleneglycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid; G6PDH, glucose-6-phosphate dehydrogenase; Hepes, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; 4-HS, 4-hydroxystilbene; LDH, L-lactate dehydrogenase; Mes, 2-[N-morpholino]ethanesulfonic acid; 4-MN, 4-methoxy-α-naphthol; Tris, tris(hydroxymethyl)aminomethane.

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from Jansen Chimica (Beerse, Belgium). Its purity was checked by elemental analysis and mass spectroscopy. Dioxane and  $\text{H}_2\text{O}_2$  were purchased from Merck (Darmstadt, F.R.G.). 4-Methoxy- $\alpha$ -naphthol (4-MN) from Aldrich-Chemie (Steinheim, F.R.G.) and 4-aminoantipyrine (4-AAP) from Sigma (St. Louis, Mo., U.S.A.). All the other chemicals used in this work were obtained from various commercial suppliers, and were of the highest purity available.

#### *Callus cultures and subcellular fractionation*

Grape-vine (*Vitis vinifera* cv. Gamay) callus cultures were kindly supplied by Drs. J. C. Pech and A. Latché (ENSA, Toulouse, France) and cultured on a Murashige and Skoog salt basal medium supplemented with vitamins, sucrose and hormones [10]. Calli were grown in 250 ml flasks containing 100 ml of medium for 20 days at 25 °C, and with a 14/10 h fluorescent light photoperiod of  $24 \mu\text{E m}^{-2} \text{s}^{-1}$ .

In order to establish the subcellular localization of peroxidases capable of oxidizing 4-HS, grape-vine calli were homogenized with a mortar and pestle, and soluble, membrane and cell wall fractions were isolated as previously described [10]. The cell wall pellet was considered to be a purified cell wall fraction on the basis of the absence of protoplast (glucose-6-phosphate dehydrogenase [EC 1.1.1.49], Mg-stimulated ATPase [EC 3.6.1.3] and anthocyanin) markers [10].

Ionically bound grape-vine peroxidases attached to membranes and cell wall fractions were solubilized by treatment with 1 M KCl [10], and dialyzed overnight at 4 °C against 50 mM Tris-HCl buffer, pH 7.5, containing activated charcoal in order to remove anthocyanins from protein fractions [10].

#### *Suspension cell cultures and protoplasts isolation*

Suspension-cultured cells were established from growing callus and cultured at 25 °C up to 90% packed cell volume (16–18 days) in 250 ml flasks, containing 100 ml of the above described culture medium, under a 14/10 h fluorescent light photoperiod regime of  $8 \mu\text{E m}^{-2} \text{s}^{-1}$ .

Protoplasts were obtained from 16–18 day old cultured cells. For this, about 15 ml of packed cells were first rinsed three times with 0.7 M mannitol, 5 mM KCl, 2 mM  $\text{CaCl}_2$ , and 25 mM K-Mes buffer,

pH 5.8, containing 0.4% (w/v) polyethyleneglycol 6000 (mannitol buffer). The cells were then incubated for 30, 60 and 90 min at 25 °C under gentle shaking in the mannitol buffer in the presence of 0.5% (w/v) pectinase (Rohament P5) and 1.0% (w/v) cellulase (Onozuka R10). The released protoplasts were filtered through a cellulose gauze and washed three times with mannitol buffer. The protoplast yield was about 40%. As a control, protoplasts were stained with calcofluor white to check complete removal of the cell wall [11].

#### *Vacuole isolation and purification*

Starting from protoplasts, vacuoles were isolated according to Aerts and Schram [12] using the polybase-induced lysis procedure with some modifications. In this case, about 5 ml of protoplasts were suspended in 5 ml 10% Ficoll in 25 mM Tris, 25 mM Mes, 1 mM  $\text{CaCl}_2$ , 0.65 M sorbitol, 0.1% bovine serum albumin, pH 7.0 (sorbitol buffer). The suspension was overlaid with 10 ml 7.5% Ficoll in sorbitol buffer, containing additionally 4 mg/ml DEAE-dextran and 1 mM EGTA, and with 5 ml of 5% and 2% Ficoll in sorbitol buffer containing 1 mM EGTA, 1 mg/ml and 3 mg/ml dextran- $\text{SO}_4$ , respectively. Finally, this Ficoll step gradient was overlaid with 3 ml of sorbitol buffer. The tube was centrifuged for 45 min at  $2000 \times g_{\text{max}}$  and the vacuoles floating at the 0–2% Ficoll interface were harvested using a Pasteur pipette. In order to purify vacuoles, the suspension was thoroughly mixed and brought to more than 10% Ficoll by adding 20% Ficoll in sorbitol buffer. The suspension was overlaid with 10%, 5% and 0% Ficoll in the same buffer and the centrifugation was repeated. Purified vacuoles were collected from the 0–5% Ficoll interface. The yield of intact vacuoles was determined by measuring the  $\alpha$ -mannosidase content of the final vacuolar preparations and of the starting amount of protoplasts. The yield normally found was about 20%.

#### *Cell, protoplast and vacuole peroxidase fractions*

Peroxidase fractions were obtained by homogenization of the cells (previously washed three times with mannitol buffer), protoplasts or vacuoles, in 2.5% (w/v) sucrose in 0.1 M Na-Mes buffer, pH 6.5, as described [10]. Cell peroxidase fractions were then purified by chromatography on in-

soluble polyvinyl-polypyrrolidone (PVPP, Sigma, St. Louis, Mo., U.S.A.), and dialyzed overnight against buffered, activated charcoal [10].

#### *Determination of enzymatic activities and anthocyanins*

The assay of peroxidase activity using 4-MN was carried out as described by Ferrer *et al.* [13], while the assay of peroxidase activity using 4-hydroxystilbene was carried out according to Calderón *et al.* [14]. Unless otherwise stated, the spectrophotometric assay for 4-HS peroxidase was typically performed at 25 °C on an assay medium containing 0.25 mM 4-HS (27.5 µl of a 10 mM 4-HS stock in HPLC-grade methanol), 5 mM H<sub>2</sub>O<sub>2</sub>, and 15% (v/v) dioxane in 28.3 mM Na-phosphate, 28.3 mM Na-borate, 28.4 mM Na-citrate (85 mM PBC) buffer at variable pH (see Results and Discussion).

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) and L-lactate dehydrogenase (LDH, EC 1.1.1.27) were assayed spectrophotometrically by monitoring the changes in optical density at 340 nm [15, 16]. Acid phosphatase (EC 3.1.3.2) and  $\alpha$ -mannosidase (EC 3.2.1.24) were assayed as previously described [17]. Anthocyanins were extracted and measured as described by García-Florenciano *et al.* [10].

#### *Isoelectrofocusing and zymographic stain of peroxidase isoenzymes*

Isoelectrofocusing of peroxidase isoenzymes was performed at 4 °C using a 2103 PS LKB power supply. Polyacrylamide gels for disc-IEF were prepared and polymerized according to Calderón *et al.* [18]. The protein was focused as already described [18]. To stain the peroxidase isoenzymes capable of oxidizing 4-HS, a staining solution buffered at pH 3.0 was prepared, based on that described elsewhere [18]. Staining was carried out at 25 °C for 20 min, and gels were destained in water. Controls were performed in the absence of 4-HS.

Staining of peroxidase isoenzymes with 4-MN was performed according to Ferrer *et al.* [13], using 4-MN at a concentration of 1 mM. The gels were destained in water, and kept at 4 °C in the dark. The 4-MN-stained gels were finally scanned at 620 nm using a Joyce-Loebl MK II scanner densitometer equipped with a linear transport.

## Results and Discussion

### *Subcellular localization of grape-vine peroxidase isoenzymes capable of oxidizing 4-HS*

The fractionation of a heterotrophic strain of Gamay grape-vine cells cultured in a solid medium at day 20 of culture reveals that most of the 4-MN peroxidase activity (450 nkat g FW<sup>-1</sup>, 69.0%) is located in the soluble (non-sedimentable) fraction. The remainder is associated with both the cell wall (200 nkat g FW<sup>-1</sup>, 30.6%) and the Mg<sup>2+</sup>-pelleted membrane (2 nkat g FW<sup>-1</sup>, 0.4%) fraction. Similar to 4-MN peroxidase activity, 4-HS peroxidase activity is mainly (about 90.2%, 570 nkat g FW<sup>-1</sup>) located in non-sedimentable fractions, only 9.2% of the total activity being located in the cell walls (58 nkat g FW<sup>-1</sup>).

Although, in outline, the subcellular localization of peroxidase activity was similar when using both substrates, some differences appeared when the ratio of 4-HS/4-MN peroxidase activity was calculated for each subcellular fraction. Thus, the ratio (*r*) was higher for the membrane (*r* = 2.0) and the soluble (*r* = 1.27) fractions than for the cell wall fraction (*r* = 0.29) isolated from Gamay cell cultures.

These observations suggest the presence in the several subcellular fractions of a population of different peroxidase isoenzymes, probably with a different capability for oxidizing 4-HS. With this in mind, the isoperoxidase patterns of soluble, membrane and cell wall fractions isolated from Gamay cell cultures were analyzed by protein isoelectrofocusing. The results for soluble peroxidase activity are shown in Fig. 1A, and illustrate a complex isoperoxidase pattern, characterized by the presence of only one isoperoxidase (B<sub>5</sub>) capable of oxidizing 4-HS to any great extent, although this was also oxidized to a minor extent by the isoenzyme B<sub>4</sub> (Fig. 1A).

This high substrate specificity of soluble isoperoxidases for 4-HS was also shown by isoperoxidases isolated from both cell wall and membrane fractions. Thus, of all the isoperoxidases found in the several subcellular fractions, only the B<sub>4</sub> and B<sub>5</sub> isoperoxidases were capable of oxidizing 4-HS. The high 4-HS/4-MN peroxidase activity ratio found in membranes and soluble fractions from Gamay cell cultures is apparently determined by

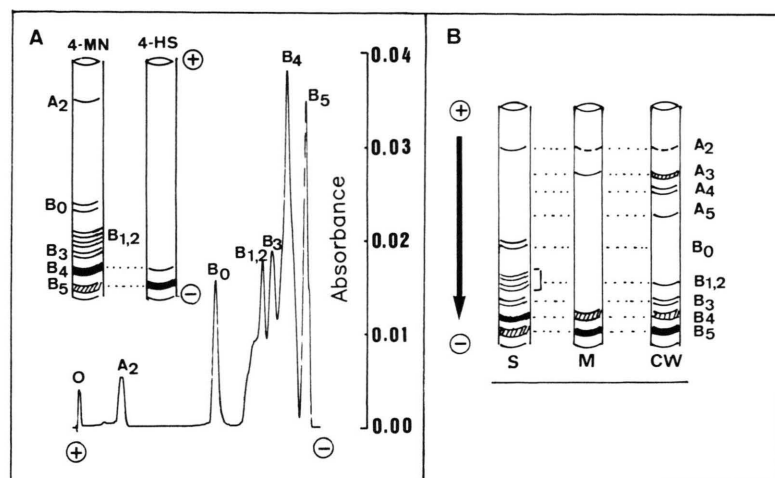


Fig. 1. A) Densitometric recording of soluble peroxidase isoenzymes stained with 4-MN, and comparative zymographic pattern of soluble peroxidase activity obtained from callus-derived cells, separated by isoelectrofocusing, and stained with 4-MN and 4-HS. O, origin. B) Zymographic patterns of soluble (S), membrane-bound (M) and cell wall-bound (CW) peroxidase activity obtained from callus-derived cells and stained with 4-MN. The arrow indicates the direction of protein migration during isoelectrofocusing. Comparative banding intensity is indicated by the degree of shading.

the enrichment of the B<sub>4</sub> and B<sub>5</sub> isoperoxidases in both soluble and membrane fractions (Fig. 1B).

From the above results, it can be expected that most of the isoperoxidases capable of oxidizing 4-HS are located in soluble fractions from cell homogenates. Three main subcellular origins have been proposed for this soluble peroxidase activity in plant cells: i) cell wall and other apoplastic spaces [19, 20], ii) cytoplasm [20], and iii) vacuole [10, 21].

Since these several subcellular origins for the soluble peroxidase activity can be differentiated by protoplast and vacuole preparation, an attempt was made to isolate protoplasts and vacuoles and to analyze their peroxidase isoenzyme patterns.

For this purpose, protoplasts were prepared from suspension-cultured cells. Protoplasts were obtained with a high yield (about 40%) starting

from 30 min of exposure to the digesting enzymes. Results shown in Table I suggest that, although 8.5% of the total 4-HS peroxidase activity is located in cell walls, most of 4-HS peroxidase activity (about 91.5%) can be recovered in protoplasts after digestion of the cell walls of suspension-cultured cells. Furthermore, the 4-HS/4-MN peroxidase activity ratio for the peroxidase located in protoplasts was close to that found in the supernatants of the centrifugation of homogenates of suspension-cultured cells (Table I).

Vacuoles were isolated by disruption of the protoplast plasma membrane by the action of the polybase DEAE-dextran procedure [22]. As with *Petunia* protoplasts [12], floatation in a polybase lysis gradient resulted in purer vacuolar fractions than those obtained by centrifugation down the gradient as described by Boudet *et al.* [22]. At this

Table I. Effect of the maceration time on protoplast yield, calculated on the basis of the total content of both anthocyanins and glucose-6-phosphate dehydrogenase; the residual level of peroxidase activity (nkat ml<sup>-1</sup>) in protoplasts measured using 4-MN and 4-HS, and the ratio of peroxidase activity using 4-HS and 4-MN as substrates.

	Protoplast yield		Peroxidase		Ratio
	Anthocyanins neq	Glucose-6-P dehydrogenase nkat	4-HS	4-MN	
Cells	2830 (100)	13.73 (100)	152.0	117.0	1.30
Protoplasts:					
30 min	1160 (41)	5.49 (40)	55.6	41.0	1.36
60 min	1075 (38)	5.22 (38)	52.8	38.3	1.37
90 min	1047 (37)	4.94 (36)	50.8	35.5	1.43



stage, contamination by protoplasts was low (the vacuole/protoplast ratio was always greater than 10), and further centrifugation on Ficoll gradients proved to be suitable for the purification of vacuoles from cytoplasmic constituents. In this context, vacuole contamination by the cytoplasmic enzyme marker G6PDH was reduced from 9% (3.15% of total G6PDH initially found in protoplasts was present in vacuoles for a vacuole yield of 35%) to about 3% (0.57% of total G6PDH initially found in protoplasts was present in vacuoles for a vacuole yield of about 20%) after Ficoll gradient purification.

Similar results were obtained when LDH was used as a negative marker enzyme of the vacuolar fractions. In this case, 1.8% of total LDH activity found in protoplasts was present in the final vacuole preparation.

Table II shows that, similar to acid phosphatase and anthocyani(di)ns, most of the peroxidase activity located in protoplasts is located in vacuoles, as shown by  $\alpha$ -mannosidase when used as an enzyme marker of the vacuolar sap [23] to calculate vacuole yields. Likewise, the peroxidase isoenzyme patterns of protoplasts (Fig. 2 A) were identical to that obtained for the peroxidase activity located in vacuoles (Fig. 2 B). In both cases, the zymographic patterns show the major presence of the isoperoxidase B<sub>5</sub>. Therefore, it can be concluded that the 4-HS-oxidizing isoperoxidase B<sub>5</sub> is mainly located in the vacuolar sap.

Similar conclusions have been reached for the localization of the B<sub>5</sub> isoperoxidase in vacuoles of Gamay cells using enzyme-binding probes and cytochemical procedures [10].

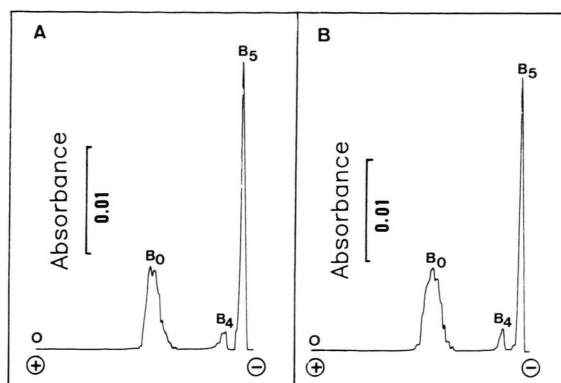


Fig. 2. Densitometric recording of the soluble peroxidase activity located in protoplasts (A) and vacuoles (B), separated by isoelectrofocusing in 3.5–10 pH gradients, and stained with 4-MN.

#### *Functionality of the 4-HS oxidizing system in cultured cells*

Gamay cell suspension cultures originated from the berries of a teinturier cultivar which accumulated mainly peonidin and cyanidin, as opposed to grape cultivars in which malvidin derivatives are predominant [24, 25]. Thus, the particular expression of the phenolic metabolism which exists in the whole fruit is conserved in the *in vitro* culture as regards the various forms of anthocyani(di)ns which accumulated [24, 25]. In this context, the localization of 4-HS oxidizing isoperoxidases in vacuoles raises the question of their functionality, since anthocyani(di)ns are mainly located in vacuoles (Table II), and these compounds are long-known strong antioxidant agents [26, 27]. Besides, the pH

Table II. Enzymatic activities, expressed in nkat, in protoplast and vacuolar preparations (obtained with a yield of 20% over protoplasts) from *Vitis vinifera* cv. Gamay cell suspension cultures and relative enzyme activities in vacuoles, assuming  $\alpha$ -mannosidase to be 100% vacuolar.

Enzymatic activity	Protoplast preparation	Vacuole preparation	% in vacuoles
$\alpha$ -Mannosidase	1.91 (100.00)	0.41 (21.46)	100.0
Acid phosphatase	3.71 (100.00)	0.57 (15.36)	71.6
Peroxidase	3.34 (100.00)	0.62 (18.69)	87.1
Anthocyani(di)ns (neq)	215.00 (100.00)	36.6 (17.02)	79.3

of the grape-vine vacuolar sap is strongly acid (around 3.0) [28], and this may give rise to a non-functionality of the 4-HS-oxidizing system.

For these reasons, the 4-HS-oxidizing activity of grape-vine peroxidase was analyzed as regards pH and anthocyani(di)n concentration.

The dependence on pH of the 4-HS-oxidizing peroxidase activity is shown in Fig. 3, and illustrates that maximal oxidation rates are achieved in the 4.0–5.0 pH range. Although at pH 3.0, the enzymatic activity is inhibited by about 60%, the remaining 40% seems to be sufficient for considering that the 4-HS-oxidizing system can be functional in the vacuole.

The effect of the anthocyani(di)n concentration on 4-HS peroxidase activity was complex (Fig. 4). The anthocyani(di)ns tested were peonidin (Fig. 4A), delphinidin (Fig. 4B), cyanidin (Fig. 4C) and its corresponding glycoside, cyanin (Fig. 4D). In all cases, anthocyani(di)n concentrations in the order of 50–100  $\mu\text{M}$  were capable of inhibiting the enzymatic activity by about 50%, when this activity was calculated from the time-course recording, and expressed as the steady-state oxidation rate. The inhibitory effect of anthocyani(di)ns was non-specific for 4-HS as electron donor, since a similar effect was found when 4-MN was used as peroxidase substrate. In this case, an inhibition of 50% was achieved for a cyanin concentration of 50  $\mu\text{M}$ .

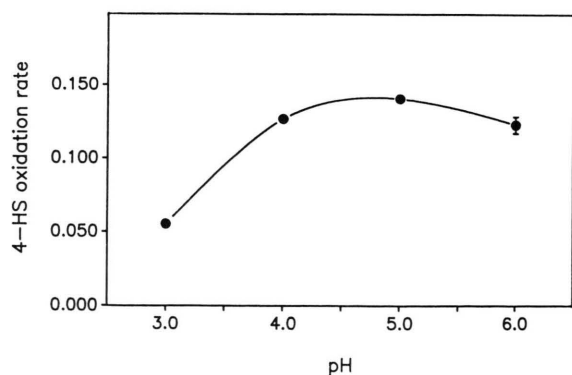


Fig. 3. Effect of the pH of the PBC buffer on the capability of Gamay grape-vine isoperoxidases to oxidize 4-HS. Bars show SE.

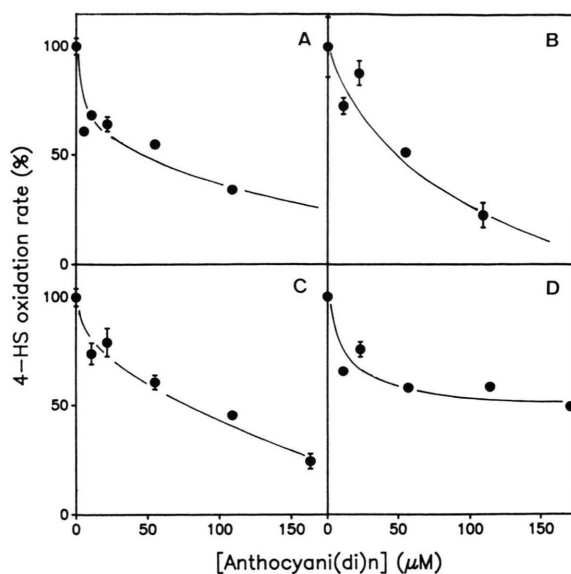


Fig. 4. Effect of peonidin (A), delphinidin (B), cyanidin (C) and cyanin (D) on the capability of grape-vine peroxidases to oxidize 4-HS at pH 4.0. Bars show SE.

These results suggest that the functionality of the 4-HS-oxidizing peroxidase system in anthocyani(di)n-containing vacuoles is doubtful, even more when the anthocyani(di)n concentration found in vacuoles of subepidermal grape-vine cells is in the order of 1–10 mM [28].

Inhibition of the 4-HS peroxidase system in grape-vine vacuoles as a consequence of anthocyanin accumulation may be partly responsible for the loss of viniferin production potential which accompanies grape berry veraison [29]. This is supported by the fact that resveratrol production [30, 31], peroxidase [32], and anthocyani(di)ns [28] are mainly located in the epidermal and the subepidermal cell layers which constitute the berry skin.

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