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# Sugar sensing and $Ca^{2+}$–calmodulin requirement in *Vitis vinifera* cells producing anthocyanins

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**Abstract**

We have previously reported that sucrose modulates anthocyanin biosynthesis in cell suspension cultures of *Vitis vinifera* L. The main role of sugar in this response does not seem to be that of general carbohydrate source for the supply of energy. In the present work, a number of pharmacological agents were used to further investigate the components of the signal transduction pathway involved in the induction of anthocyanin biosynthesis by sugar. We found that the phosphorylation of hexose by hexokinase, but not its transport, has to be taken into account for the sucrose signal transduction leading to anthocyanin accumulation. Indeed, 3-*O*-methylglucose, a glucose analog transported into cells but not phosphorylated by hexokinase, has no effect on anthocyanin production. Mannose mimics the effect of sucrose in grape cells, and mannoheptulose, a specific inhibitor of hexokinase, reduces the accumulation of anthocyanins in response to sucrose. The results with the two latter analogs are discussed. $Ca^{2+}$ channel blockers, verapamil and $LaCl_3$, which were used to investigate the role of extracellular $Ca^{2+}$, all inhibited the sugar response. $Ca^{2+}$ depletion by pretreatment with ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) also blocked the sugar response, which was partially recovered when $Ca^{2+}$ was added exogenously after $Ca^{2+}$ depletion. The use of two potent calmodulin antagonists, *N*-(6-aminohexyl)-5-chloro-1-naphtalenesulphonamide (W7) and chlorpromazine, showed that calmodulin is involved in the sugar signal transduction. A protein kinase inhibitor, 6-dimethylaminopurine (6-DMAP), and the protein phosphatase inhibitors, endothall and cantharidin, also inhibited the sugar response. The results of the present study suggest the involvement of several components of general signal transduction pathways such as $Ca^{2+}$, calmodulin, and protein kinases/phosphatases in the induction of anthocyanin biosynthesis by sugar. 

*Keywords:* *Vitis vinifera*; Vitaceae; Grapevine; Anthocyanins; Sugar sensing; Calcium; Calmodulin; Protein phosphorylation

## 1. Introduction

Anthocyanins are widely found in various plant species. They have a high potential value as natural food colorants and may exert a protective effect against atherogenesis due to their antioxidant properties on human LDL (Fauconneau, Waffo Téguo, Huguet, Barrier, Decendit & Mérillon, 1997).

Using *Vitis vinifera* cell suspension cultures, we showed that cells grown in a polyphenol-inducing medium synthesize high levels of anthocyanins, proanthocyanidins, catechins, and stilbenes i.e. the major polyphenols found in grape berries and red wine (Decendit & Mérillon, 1996; Waffo Téguo, Decendit, Krisa, Deffieux, Vercauteren & Mérillon, 1996; Krisa, Waffo Téguo, Decendit, Deffieux, Vercauteren and Mérillon, 1999). The optimal biosynthetic capabilities of these polyphenols depend on nutritional and en-

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vironmental conditions. Sucrose was found to modulate polyphenol accumulation in *V. vinifera* cell cultures. Indeed, the accumulation of anthocyanins was strongly increased (12-fold) by sucrose (0.15 M), while that of stilbenes was only slightly affected (Larronde, Krisa, Decendit, Chèze, Deffieux & Mérillon, 1998). The latter study showed that sucrose does not play a physical role, while metabolic sugars are, nevertheless, required for the induction of anthocyanin production. Moreover, a close correlation has been found between anthocyanin accumulation and the level of intracellular hexoses (glucose and fructose) in *V. vinifera* cell cultures. This phenomenon can be understood as a sugar-dependent expression of the chalcone synthase gene, as has recently been shown for the chalcone synthase gene from *Petunia* in transgenic *Arabidopsis* (Tsukaya, Ohshima, Naito, Chino & Komeda, 1991) and that from *Camellia sinensis* (Takeuchi, Matsumoto & Hayatsu, 1994).

Likewise, during ripening, grape berries accumulate sugars in the form of glucose and fructose in the vacuoles (flesh and skin), after translocation of sucrose from the leaves (Coombe, 1992; Davies & Robinson, 1996). Furthermore, an increase in the expression of several genes in the anthocyanin biosynthetic pathway (in particular, chalcone synthase) has been observed (Boss, Davies & Robinson, 1996). Indeed, anthocyanin biosynthesis increases in the skin cells of berries, while the level of stilbene phytoalexins declines. Therefore, grape berries become susceptible to fungal attack, which causes large economic losses (Jeandet, Sbaghi, Bessis & Meunier, 1995). This raises questions concerning the mechanism underlying the control of anthocyanin and stilbene accumulation under the influence of sugars in grapevine.

In plants, sugars are not only energy sources and structural components, but are also physiological signals regulating the expression of a variety of genes (Jang, Leon & Sheen, 1997). To date, relatively little is known about the perception and the transduction of the sugar signal, although hexokinase can act as a sensor for plant sugar responses including both sugar-repressible and sugar-inducible gene expression, but the hexose effect does not require the glycolytic pathway downstream of this first step (Jang et al., 1997). Two other sugar-sensing systems independent of hexokinase have been proposed in plants: a hexose transport-associated sensor and a sucrose-specific pathway involving a sucrose transporter (Smeekens & Rook, 1997). This latter mechanism did not seem to be involved in our system, because the hexoses (glucose, fructose) had a positive effect on anthocyanin accumulation similar to that of sucrose, and sucrose was undetectable in grape cells (Larronde et al., 1998). Here, the sucrose response is probably attributable to hexoses derived from hydrolyzed sucrose.

Calcium has been shown to serve as a second messenger in the signal transduction of environmental stimuli and hormones in plants (Bush, 1995; Trewavas & Malho, 1997). Two kinds of calcium stores are thought to contribute to the elevation of cytosolic $Ca^{2+}$; those of extracelluar and intracellular (vacuole, endoplasmic reticulum) origin. Calcium regulates the activities of target proteins directly or via calcium binding proteins such as the ubiquitous calmodulin, which after binding to $Ca^{2+}$ activates a number of protein kinases and other proteins in plant cells (Trewavas & Malho, 1997), and evidence has accumulated for the existence of $Ca^{2+}$-dependent protein kinases in plants (Roberts & Harmon, 1992). Moreover, it has been proposed that protein phosphatase and protein kinase are involved in sugar signal transduction in plants (Jang & Sheen, 1997; Ehness, Ecker, Godt & Roitsch, 1997).

By using a pharmacological approach, the purpose of the present study was to investigate sugar sensing and signal transduction within our model system, *V. vinifera* cell cultures, which accumulate high levels of anthocyanins under the influence of sucrose.

## 2. Results and discussion

We chose to add sucrose at day 7 of the growth period, which resulted in a large stimulation of anthocyanin accumulation 3 days later (Larronde et al., 1998).

### *2.1. Sugar sensing in grape cells accumulating anthocyanins*

First, we examined the possibility that the sugar response is due to a hexose transport-associated sensor. We tested 3-*O*-methylglucose, a glucose analog that is transported into cells but not phosphorylated by hexokinase. No apparent difference in anthocyanin production was observed between the negative control and cells treated with 3-*O*-methylglucose (0.01–0.15 M) (Table 1). The sensing mechanism in our system does not occur before hexokinase in the signaling pathway.

We investigated the effect of mannose or 2-deoxyglucose instead of sucrose (0.15 M) on anthocyanin production in grape cells. Glucose analogs are phosphorylated by hexokinase, and have been widely considered as non-metabolisable through glycolysis. As shown in Table 1, mannose (0.02–0.1 M) stimulated the accumulation of anthocyanins proportionally to the concentration added. At 0.10 M, it led to a response of 70% in comparison with sucrose. It is necessary to use a high external concentration of mannose in our model, which is in contrast to the lower

concentrations (0.002–0.010 M) sufficient to obtain a response in other systems (Klein & Stitt, 1998; Pego, Weisbeek & Smeekens, 1999). The dry weight of grape cells increased notably after the addition of mannose, as found with sucrose which is known mainly to induce an increase in the carbohydrate content without a notable effect on grape cell proliferation (Larronde et al., 1998). Moreover, no toxicity was observed for grape cells even in the presence of 0.10 M mannose. Therefore, grape cells are mannose-insensitive, so mannose-6-phosphate is probably isomerized to fructose-6-phosphate by a mannose-6-phosphate isomerase, as previously reported in the red alga *Galdieria sulphuraria* (Heilmann, Schnarrenberger & Gross, 1997) and in germinating legume seeds (MacCleary & Matheson, 1976). It is not possible to interpret the increase of anthocyanin production after adding mannose to grape cells as evidence for a role of hexokinase in sugar-sensing. When 2-deoxyglucose was used, it was toxic to grape cells from a concentration as low as 0.5 mM; at concentrations lower than this threshold no change in anthocyanin production was observed (result not shown). This compound, which leads to a major metabolic disruption, seems unsuitable as a tool to investigate the involvement of hexokinase in sugar-sensing (Klein & Stitt, 1998).

To support the involvement of hexokinase in our model, we sought to determine the effect of a specific hexokinase inhibitor such as mannoheptulose (Jang et al., 1997). As can be seen in Table 1, grape cell suspensions treated with mannoheptulose showed a reduction of anthocyanin production reaching (at 5 mM) 46% of the sucrose control. No effect of this competitive inhibitor was observed on anthocyanin production in the control without added sucrose. Mannoheptulose did not notably affect cell growth. It may seem surprising that 5 mM mannoheptulose acting on hexokinase by a competitive mechanism with a lower affinity can counteract the effect of 150 mM sucrose, leading to high intracellular concentration of hexoses (Larronde et al., 1998). However, metabolic compartmentation is primordial; hexoses are stored within the vacuoles of grape cells, whereas hexokinase is located in the cytosol. Moreover, although vacuolar invertases are involved in hexose accumulation in grape berries (Davies & Robinson, 1996), another enzyme, sucrose synthase, may also be involved in the breakdown of sucrose. Sucrose synthase is largely dominant in *Lycopersicon esculentum* fruit (Wang, Sanz, Brenner & Smith, 1993) and is present at high activities in rapidly growing tissues (Geigenberger & Stitt, 1993). The sucrose synthase pathway detected in grape berries by Hawker (1969) allowing the bypass of the hexokinase step may play a significant role in grape cells. Therefore, the modulation of the hexokinase pathway by mannoheptulose is plausible.

These results suggest that the phosphorylation of hexoses via hexokinase has to be taken into account for the sucrose signal transduction leading to anthocyanin accumulation.

### 2.2. Sugar-stimulated anthocyanin biosynthesis is mediated by calcium

Calcium channel blockers have been widely used to demonstrate the involvement of calcium in biochemical responses in several mammalian and plant systems

Table 1
Effects of sucrose analogs, 3-*O*-methyl glucose and mannose, and of hexokinase inhibitor mannoheptulose (MH) on anthocyanin accumulation in *Vitis vinifera* cells[a]

| Treatment | Dry weight | s.d. | Anthocyanin production | s.d. |
|---|---|---|---|---|
| Control | 40 | 1 | 25 | 1 |
| Sucrose 150 mM | 100 | 1 | 100 | 8 |
| 3-O-M-G 10 mM | 37 | 1 | 14 | 1 |
| 3-O-M-G 50 mM | 40 | 1 | 19 | 1 |
| 3-O-M-G 100 mM | 44 | 1 | 17 | 1 |
| 3-O-M-G 150 mM | 47 | 1 | 20 | 1 |
| Mannose 20 mM | 48 | 1 | 29 | 1 |
| Mannose 40 mM | 57 | 1 | 34 | 1 |
| Mannose 60 mM | 64 | 2 | 47 | 1 |
| Mannose 80 mM | 76 | 3 | 51 | 1 |
| Mannose 100 mM | 84 | 1 | 72 | 1 |
| MH 2 mM | 41 | 1 | 24 | 1 |
| Sucrose 150 mM + MH 2 mM | 91 | 2 | 73 | 1 |
| Sucrose 150 mM + MH 5 mM | 90 | 3 | 56 | 1 |
| Mannitol 150 mM | 51 | 2 | 34 | 1 |

[a] Values are expressed as means $\pm$ s.d. ($n = 3$) relative to experiment with 0.15 M sucrose added, in which dry weight and anthocyanin accumulation were $22 \pm 1$ g $l^{-1}$ and $269 \pm 8$ μM, respectively. Cells were harvested 3 days after treatment.

(Rampe & Triggle, 1990; Priesig & Moreau, 1994; Christie; & Jenkins, 1996).

Therefore, we investigated whether calcium was involved in the sugar signaling pathway regulating anthocyanin biosynthesis by testing various calcium antagonists in grape cell cultures. Two calcium channel blockers which inhibit $Ca^{2+}$ influx were used: verapamil, a phenylalkylamine derivative, which is the most accepted blocker of voltage-dependent calcium channels in higher plant cells (Priesig & Moreau, 1994; Mérillon, Huguet, Fauconneau & Rideau, 1995); and lanthanum chloride ($La^{3+}$), which competes externally with calcium for all plasma membrane calcium channels. Results are given in Fig. 1. The addition of verapamil to the culture medium at 50 μM inhibited by 40% the accumulation of anthocyanin in response to sucrose. The inhibition reached 60% at 500 μM. Similarly, anthocyanin induction by sucrose was affected when grape cells were treated with $LaCl_3$. The inhibition was proportional to the concentration of $La^{3+}$, the accumulation of anthocyanin relative to the sugar control being 70, 45 and 19% at 100, 200 and 500 μM, respectively. These two calcium channel blockers were active without notably affecting cell growth. On the other hand, no significant inhibition of anthocyanin accumulation was observed when these reagents were added to grape cell cultures without application of sucrose.

The divalent cation chelator, ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), which is commonly used to probe extracellular $Ca^{2+}$ involvement, also inhibited sugar-induced anthocyanin accumulation (Fig. 2). The EGTA concentration needed to obtain an inhibition of around 60% was between 2 and 5 mM, but the cell growth was slightly inhibited. Addition of $CaCl_2$ partly relieved the inhibition of anthocyanin accumulation by EGTA. We also tested whether calcium release from intracellular stores is involved in this sugar signaling pathway. Therefore, we examined the effect of ruthenium red, which is known to block endomembrane $Ca^{2+}$ channels, e.g. vacuolar voltage-dependent channels (Allen, Muir & Sanders, 1995). Incubation of grape cells with 10–50 μM ruthenium red did not affect anthocyanin production in response to sucrose (data not shown).

These experiments with well-established calcium antagonists used at concentrations similar to those in other studies indicate that calcium is involved in the sugar signaling pathway inducing anthocyanin biosynthesis in *V. vinifera* cell cultures, and suggest that the increase in cytosolic calcium occurs rather by an influx of $Ca^{2+}$ from extracellular origin across the plasma membrane than by an efflux of calcium from intracellular stores. Likewise, cytosolic $Ca^{2+}$ has been reported to be involved in the sugar-inducible expression of genes coding for sporamin and β-amylase of sweet potato (Ohto, Hayashi, Isobe & Nakamura, 1995).

### 2.3. Calmodulin is involved in the sugar transduction pathway regulating anthocyanin accumulation

We tested the effects of calmodulin antagonists which have been used to demonstrate an involvement of calmodulin in a number of plant responses (Priesig

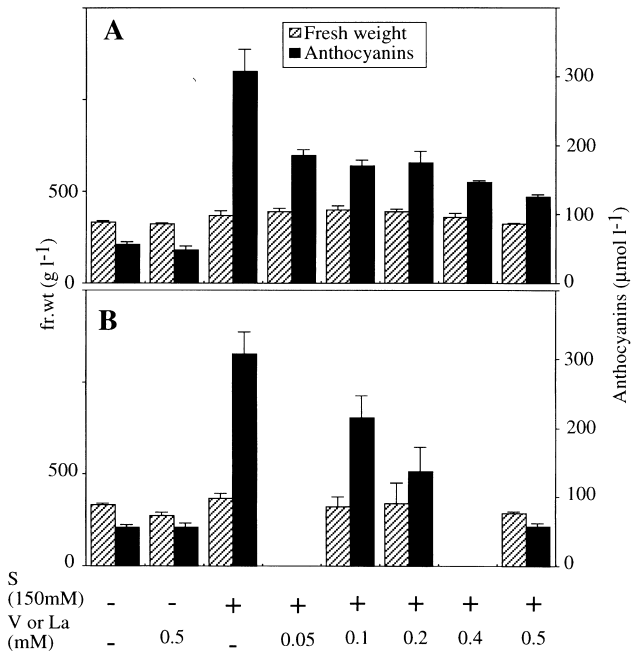


Fig. 1. Effect of calcium channel blockers on growth and sucrose-induced anthocyanin production of grape cells grown in IM2 medium. Sucrose (S) and drugs were added at day 7 and cells were harvested at day 10. V: Verapamil (A); La: Lanthanum chloride (B). Three replicates (error bars show s.d.).

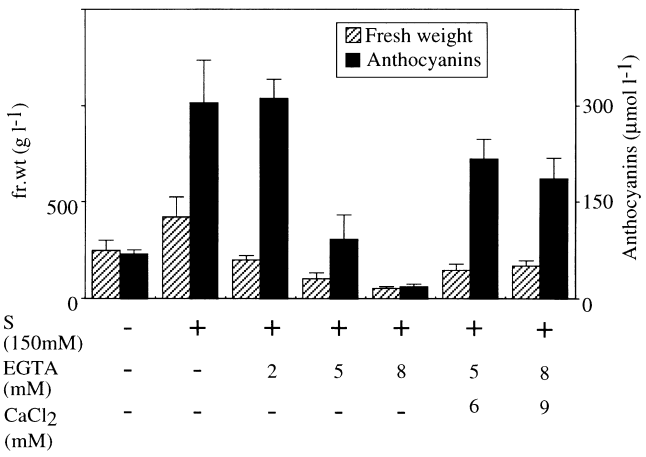


Fig. 2. Effect of EGTA on growth and sucrose-induced anthocyanin production of grape cells grown in IM2 medium. Sucrose (S) and EGTA were added at day 7 and cells were harvested at day 10. Three replicates (error bars show s.d.).

& Moreau, 1994; Christie & Jenkins, 1996). Chlorpromazine and *N*-(6-aminohexyl)-5-chloro-1-naphtalenesulphonamide (W7), the most selective calmodulin antagonists, prevented sugar-induced anthocyanin accumulation in grape cells (Fig. 3). These drugs were active at concentrations lower than 100 μM, without affecting cell growth. Therefore, at 50 μM, they inhibited anthocyanin production by 66 and 40%, respectively. These results suggest that in the sugar signaling pathway inducing anthocyanin biosynthesis, there is the involvement of calmodulin or $Ca^{2+}$-dependent protein kinase containing a calmodulin-like domain that is also inhibited by calmodulin antagonists (Takezawa, Patil, Bhatia & Poovaiah, 1996).

### *2.4. Effects of protein kinase and protein phosphatase inhibitors on sugar-stimulated anthocyanin biosynthesis*

6-Dimethylaminopurine (6-DMAP), identified as an inhibitor of a variety of animal protein kinases and of tobacco protein kinase (Mathieu, Sanchez, Droillard, Lapous, Laurière & Guern, 1996), was used to assess the possible involvement of protein phosphorylation in sugar signaling. When grape cells were preincubated in the presence of 6-DMAP before the addition of sucrose, the anthocyanin contents were gradually decreased with increasing inhibitor concentrations, from 35% at 150 μM to 55% at 500 μM, without any reduction in growth (Table 2).

We also tested other protein kinase inhibitors, staurosporine (0.2–0.4 μM), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7) (10–100 μM), and genistein (50–400 μM), but these inhibitors did not have any significant effect at these concentrations. Staurosporine, which is an inhibitor of broad spectrum activity, was not active in our model, perhaps because a too low concentration was used. Concerning H7 (a potent inhibitor of cyclic nucleotide-dependent protein kinase and protein kinase C) and genistein (a specific inhibitor of protein tyrosine kinase), the effectiveness of these drugs tested in a large range of concentrations suggests that these classes of protein kinase are not involved in sugar signaling. Moreover, their poor solubility may hinder their penetration into cells.

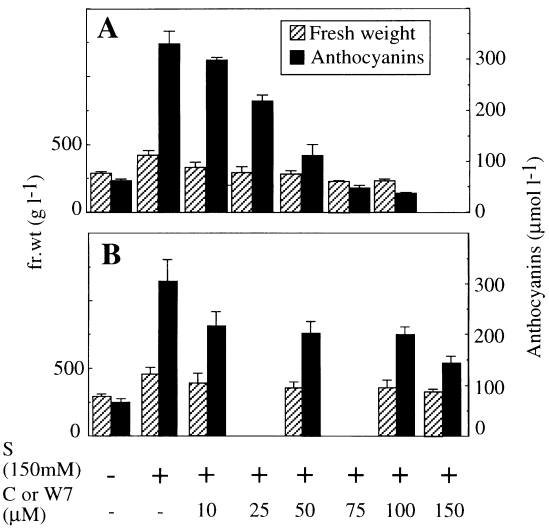


Fig. 3. Effect of calmodulin antagonists on growth and sucrose-induced anthocyanin production of grape cells grown in IM2 medium. Sucrose (S) and drugs were added at day 7 and cells were harvested at day 10. C: Chlorpromazine (A); W7 (B). Three replicates (error bars show s.d.).

Thus, it is likely that a 6-DMAP-sensitive protein kinase may participate in sugar-induced anthocyanin biosynthesis. The involvement of protein phosphorylation has already been shown in the induction of chalcone synthase gene expression by UV- and blue-light in *Arabidopsis* cells (Christie & Jenkins, 1996).

Thus, we examined the role of protein phosphatase by using specific inhibitors, endothall and cantharidin, which have been shown to be potent inhibitors of protein phosphatase type 1 and type 2A in plants and animals. These two inhibitors are particularly useful for intact plant cells since they are inexpensive and known to be taken up by suspension cultured cells (MacKintosh, Lyon & MacKintosh, 1994; Ehness et al., 1997). As shown in Table 2, the incubation of grape cells in the presence of cantharidin or endothall (1–5 μM), without addition of sucrose, did not lead to a stimulation of anthocyanin accumulation. However, these inhibitors were effective in inhibiting sucrose-stimulated anthocyanin biosynthesis. At very low concentrations, 1 and 2 μM, respectively, they inhibited anthocyanin production by 55% with a slight reduction in growth. Our results indicate that endothall- and cantharidin-sensitive protein phosphatase(s) serve as positive regulators in the sugar signal transduction pathway, resulting in stimulation of anthocyanin production. This activity may be exerted at a step different from protein kinase in the sugar transduction pathway, which may consist in a complex protein kinase/phosphatase cascade, as suggested by Kwak and Lee (1997) in the transduction of ethylene signal in the pea.

In conclusion, we have shown that hexokinase is a good candidate as sensor in sugar signaling leading to anthocyanin accumulation in grape cells. To clarify the role of hexokinase, it is necessary to investigate sugar metabolism in grape cells. Moreover, an increase in cytosolic calcium seems necessary, perhaps through the involvement of a calmodulin or $Ca^{2+}$-dependent protein kinase and a complex protein kinase/phosphatase cascade. The *V. vinifera* cell culture should, therefore, prove to be a very useful tool for studies involving the regulation of chalcone synthase gene expression by sugars.

## 3. Experimental

### 3.1. Chemicals

All reagents were purchased from Sigma, except endothall from ICN.

### 3.2. Cell culture and treatment protocol

Cell suspension of *V. vinifera* (L.) cv Gamay Fréaux var. Teinturier (Vitaceae) was maintained as described previously (Decendit & Mérillon, 1996) in a culture medium containing B5 macroelements (Gamborg, Miller & Ojima, 1968), microelements (Murashige & Skoog, 1962), vitamins (Morel, 1970) and supplemented with 58 mM sucrose, 250 mg $l^{-1}$ casein hydrolysate, 0.54 μM 1-naphthaleneacetic acid and 0.93 μM kinetin.

For experimental purposes, we inoculated a 7-day-old cell suspension into an induction medium (IM2) at a 1:8 (v/v) ratio (Larronde et al., 1998). This was similar to the maintenance medium, but also contained 2 mM $(NH_4)_2SO_4$, 2.2 mM $NaH_2PO_4$ and 2 mM $MgSO_4$. Sucrose was dissolved in water, and sterilized by filtration (0.22 μm) before being added to 7-day-old cultures. Thirty minutes before the application of sucrose, cells were treated with the agonist/antagonist or the sugar analog. Mannoheptulose, mannose, 3-*O*-methylglucose, EGTA, endothall, 6-DMAP, W7, chlorpromazine, and ruthenium red were dissolved in water. Verapamil, genistein, H7, cantharidin, $LaCl_3$ were dissolved in methanol. Staurosporine was dissolved in DMSO. All reagents were filter-sterilized. Control cultures received the corresponding vehicle solvent. More details are included in Section 2. Cells were harvested after 3 days incubation by vacuum filtration and were rapidly washed with cold distilled water, weighed and stored at −20°C until analysis.

### 3.3. Quantification of anthocyanins

Freeze-dried cells (40 mg) were extracted with methanol–0.32 M HCl (85:15, v/v) overnight at 4°C. Absorbance of the anthocyanin extract was measured at 535 nm. Anthocyanin content was calculated according to the molar extinction coefficient ($\log \varepsilon$ 4.53) of peonidin-3-*O*-β-glucoside purified from cultured grape cells.

The three major anthocyanin monoglucosides in grape cells were identified by co-chromatography with authentic standards using HPLC analysis and by spectrometric methods (Krisa et al., 1999). These monoglucosides are cyanidin-3-*O*-β-glucoside (17%), peonidin-3-*O*-β-glucoside (70%) and malvidin-3-*O*-β-glucoside (13%), which together account for 90% of the total anthocyanins. No significant change in the relative amounts of anthocyanins was observed in the different experiments.

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Table 2
Effects of the protein kinase inhibitor 6-dimethylaminopurine (6-DMAP) and protein phosphatase inhibitors, cantharidin and endothall, on anthocyanin accumulation in *Vitis vinifera* cells[a]

| Treatment | Dry weight | s.d. | Anthocyanin production | s.d. |
|---|---|---|---|---|
| Control | 38 | 1 | 24 | 1 |
| Sucrose 150 mM | 100 | 1 | 100 | 1 |
| Sucrose 150 mM + 6DMAP 150 μM | 73 | 2 | 66 | 1 |
| Sucrose 150 mM + 6DMAP 250 μM | 68 | 7 | 48 | 1 |
| Sucrose 150 mM + 6DMAP 350 μM | 71 | 1 | 57 | 1 |
| Sucrose 150 mM + 6DMAP 500 μM | 71 | 1 | 45 | 1 |
| Cantharidin 1 μM | 34 | 1 | 25 | 1 |
| Cantharidin 2 μM | 37 | 1 | 25 | 3 |
| Cantharidin 5 μM | 32 | 1 | 23 | 1 |
| Sucrose 150 mM + Cantharidin 1 μM | 73 | 1 | 45 | 12 |
| Sucrose 150 mM + Cantharidin 2 μM | 55 | 1 | 29 | 1 |
| Sucrose 150 mM + Cantharidin 5 μM | 47 | 1 | 14 | 1 |
| Endothall 1 μM | 32 | 1 | 14 | 1 |
| Endothall 2 μM | 31 | 1 | 11 | 1 |
| Endothall 5 μM | 28 | 1 | 8 | 1 |
| Sucrose 150 mM + Endothall 1 μM | 88 | 2 | 110 | 1 |
| Sucrose 150 mM + Endothall 2 μM | 80 | 2 | 48 | 1 |
| Sucrose 150 mM + Endothall 5 μM | 64 | 1 | 18 | 1 |

[a] Values are expressed as means ± s.d. ($n = 3$) relative to experiment with 0.15 M sucrose added, in which dry weight and anthocyanin accumulation were $23 \pm 1$ g $l^{-1}$ and $258 \pm 2$ μM, respectively. Cells were harvested 3 days after treatment.

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