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## Chapter 3

### Gibberellins, jasmonate and abscisic acid modulate the sucrose-induced expression of anthocyanin biosynthetic genes in *Arabidopsis*

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#### Abstract

Anthocyanins are secondary metabolites, which play an important role in the physiology of plants. We investigated the interplay between sucrose and plant hormones in the expression of gene coding for anthocyanin biosynthetic enzymes in *Arabidopsis* seedlings. The expression pattern of 14 genes involved in the anthocyanin biosynthetic pathway, including two transcription factors (*PAP1*, *PAP2*), was analyzed by real-time RT-PCR in *Arabidopsis* seedlings treated with sucrose and plant hormones. The sucrose-induction of the anthocyanin synthesis pathway was repressed by the addition of gibberellic acid whereas jasmonate and abscisic acid had a synergic effect with sucrose. The *gai* mutant is insensitive to gibberellin-dependent repression of dihydroflavonol reductase. This would seem to prove that *GAI* signalling is involved in the cross-talk between sucrose and gibberellin in wild-type *Arabidopsis* seedlings. On the other hand, the inductive effect of sucrose was not strictly ABA-mediated. Sucrose induction of anthocyanin genes requires the *COI1* gene, but not *JAR1*, which suggest a possible convergence of the jasmonate- and sucrose- signalling pathways. The results suggest the existence of a cross-talk between the sucrose and hormone signalling pathways in the regulation of the anthocyanin biosynthetic pathway.

#### Introduction

Flavonoids represent a large class of secondary plant metabolites, of which anthocyanins are the most conspicuous, due to the wide range of chemical structures derived from their synthesis. Besides providing pigmentation in flowers, fruits, seeds and leaves, anthocyanins have other important functions in plants. In petals, they attract pollinators, whereas in seeds and fruits anthocyanins can aid seed dispersal. Anthocyanins and flavonoids can also be important as feeding deterrents and as a protection against UV irradiation damage (Winkel-Shirley, 2001). Anthocyanins are considered as antioxidant molecules (Gould et al., 2002) and protect plants from damage by reactive oxygen species (Nagata et al., 2003). These properties make them interesting food ingredients for human and animal nutrition.

The involvement of anthocyanins in such diverse and important functions raises questions about how these compounds are synthesized and how their synthesis is regulated. The enzymes involved in anthocyanin biosynthesis have been characterised in several plant species including *Arabidopsis* (Shirley et al., 1995; Barthi and Khurana, 1997). The basal level of anthocyanins can be modulated by various stimuli. One important environmental factor in anthocyanin synthesis is light (Cominelli et al., 2007). In addition, phosphate limitation, cold stress and sugar addition can enhance anthocyanin accumulation induced by light (Hara et al., 2003; Lea et al., 2007).

Sugars are an important source of energy and carbon skeletons for plant growth and development, but they also act as signalling molecules whose transduction pathways may influence

developmental and metabolic processes (Smeekens, 2000; Rolland et al., 2006). Sugar signalling modulates various processes such as photosynthesis, nutrient mobilization and allocation, and it also stimulates the growth of sink tissues (Koch, 1996; Rolland et al., 2002). Many jasmonate, abscisic acid, stress-inducible, and pathogenesis-related genes are also co-regulated by sugars (Reinbothe et al., 1994; Sadka et al., 1994).

Sugar-induced anthocyanin accumulation has been observed in many plant species. In petunia, sugars were shown to be required for the pigmentation of developing corollas (Weiss, 2000), while in grape berry skin sugars were found to induce most of the genes involved in anthocyanin synthesis (Boss et al., 1996; Gollop et al., 2001; Gollop et al., 2002). Moreover, a sucrose-specific induction of anthocyanin biosynthesis was also recently demonstrated in Arabidopsis seedlings (Teng et al., 2005, Solfanelli et al., 2006).

Hormones and sugars interact or cross-talk to form a complex network of overlapping signalling, which coordinate overall plant growth and development (Loreti et al., 2000; Smeekens, 2000; Rolland et al., 2006). The influence of exogenous plant growth regulators on anthocyanin accumulation is, however, not fully understood. Jasmonate (JA) has a clear inductive effect on anthocyanin synthesis, as demonstrated by some experimental evidence in various plant species and tissues (see Table 1 for references).

**Table 1.** Bibliographic overview of the effects of plant hormones on the synthesis of anthocyanins.

	Enhancing effects	Inhibiting effects
<b>Gibberellins</b>	<i>Petunia flowers</i> (Moalem-Beno et al., 1997; Weiss et al., 1995); <i>Periwinkle flowers</i> (Ohlson and Berglund, 2001); <i>Hyacinthus sepals</i> (Hosokawa, 1999)	<i>Strawberry fruit</i> (Martinez et al., 1996); <i>Maize leaves</i> (Kim et al., 2006); <i>Carrot cell cultures</i> (Ilan and Dougal, 1992; Ilan et al., 1994); <i>Low phosphate grown Arabidopsis</i> (Jiang et al., 2007)
<b>Cytokinins</b>	<i>Maize seedlings</i> (Piazza et al., 2002); <i>Arabidopsis seedlings</i> (Deikman and Hammer, 1995; Laxmi et al., 2006); <i>Strawberry cell cultures</i> (Mori et al., 1994a)	<i>Maize leaves</i> (Kim et al., 2006); <i>Arabidopsis seedlings</i> (Wade et al., 2003)
<b>Ethylene</b>	<i>Maize leaves</i> (Kim et al. 2006); <i>Grape berry skin</i> (El-Kereamy et al., 2003)	
<b>Auxin</b>	<i>Strawberry cell cultures, high auxin treatment</i> (Mori et al., 1994)	<i>Strawberry cell cultures</i> (Mori et al., 1994); <i>Grape berry skin</i> (Ban et al., 2003; Jeong et al., 2004)
<b>ABA</b>	<i>Maize leaves</i> (Kim et al., 2006); <i>Grape berry skin</i> (Jeong et al., 2004; Mori et al., 2005; Ban et al., 2003); <i>Maize kernels</i> (Paek et al., 1997; Hattori et al., 1992)	<i>Grape berry skin</i> (Han et al., 1996); <i>Petunia flowers</i> (Weiss et al., 1995)
<b>Jasmonate</b>	<i>Strawberry fruit</i> (Ayala-Zavala et al., 2005); <i>Tulip bulbs</i> (Saniewski et al., 1998b; Saniewski et al., 2004); <i>Arabidopsis seedlings</i> (Devoto et al., 2005; Chen et al., 2007); <i>Maize leaves</i> (Kim et al., 2006); <i>Soybean plants</i> (Franceschi and Grimes, 1991); <i>Carrot cell cultures</i> (Sudha and Ravishankar, 2003); <i>Kalanchoe cell cultures</i> (Saniewski et al., 2003); <i>Potato cell cultures</i> (Plata et al., 2003); <i>Apple fruit</i> (Rudell et al., 2002); <i>Peach shoots</i> (Saniewski et al., 1998a); <i>Petunia flowers</i> (Tamari et al., 1995)	

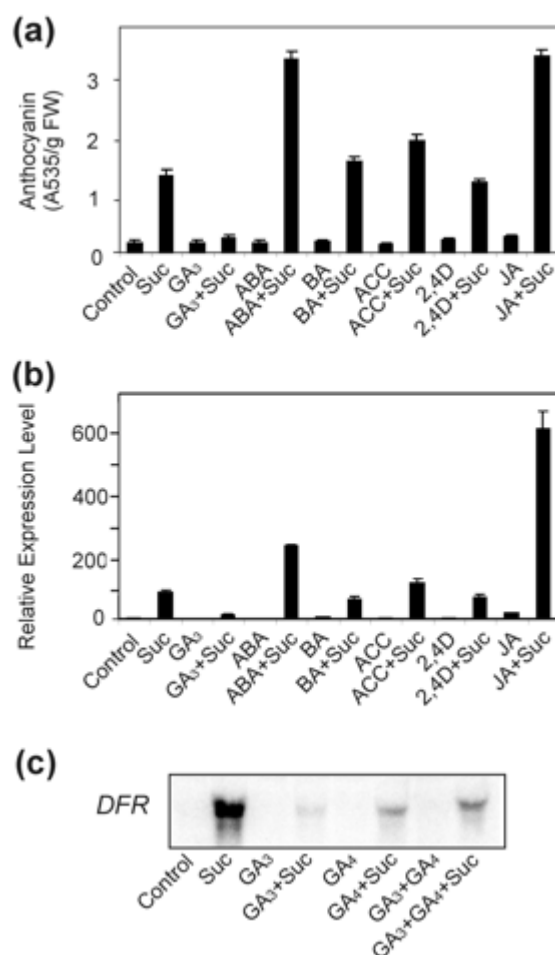
In addition, ethylene appears to have a univocally positive effect on anthocyanin accumulation, whereas the results are contradictory for gibberellins, which appear to have a positive role on flowers while playing a repressive role on various other plant tissues (Table 1). The published evidence on the effects of ABA, auxin, and cytokinins is contradictory (Table 1). Most of these studies, however, do not analyse the effects of plant hormones in terms of genes, apart from the work of Devoto et al. (2005) who showed that JA could induce the expression of some anthocyanin-related genes such as *chalcone synthase*, *anthocyanidin synthase* and *leucoanthocyanidin dioxygenase* in *Arabidopsis*.

In a previous work, Solfanelli et al. (2006) described the sucrose-specific induction of anthocyanin biosynthesis in *Arabidopsis* seedlings. In this study, we investigated the interaction between sucrose and plant hormones. The results showed that, while gibberellic acid inhibited the sucrose-induction of most of the genes involved in the anthocyanin pathway, jasmonate and abscisic acid had a synergic effect with sucrose, enhancing the induction of these genes.

## Results

### Effects of different hormones on anthocyanin synthesis

Sucrose increases anthocyanin content in *Arabidopsis* seedlings (Solfanelli et al., 2006; Teng et al., 2005). In order to gain additional clues regarding sucrose regulation, we tested the effect of hormones on the sucrose-induction of anthocyanin accumulation in *Arabidopsis* seedlings. Hormones alone were unable to affect anthocyanin levels (Figure 1a). Treatments with benzyl adenine (BA), auxin (2,4D), and 1-aminocyclopropane-1-carboxylic acid (ACC), in combination with sucrose, did not significantly influence anthocyanin contents (Figure 1a). Deikman and Hammer (1995) observed a complex regulation of anthocyanin biosynthetic genes triggered by cytokinins, but other experiments have discounted a possible role of cytokinins on anthocyanin accumulation in *Arabidopsis* (Wade et al., 2003), in agreement with our results (Figure 1a). Interestingly, when gibberellic acid ( $GA_3$ ) was supplied together with sucrose, we observed an inhibiting effect on the sucrose-induction of anthocyanins (Figure 1a). On the other hand, the addition of jasmonate (JA) and abscisic acid (ABA) to sucrose was very effective in enhancing anthocyanin accumulation (Figure 1a). We also measured the mRNA level of dihydroflavonol reductase (*DFR*), a sucrose-induced gene involved in anthocyanin synthesis (Solfanelli et al., 2006). The results were in accordance with the anthocyanin content, indicating that  $GA_3$  inhibited the sucrose-induction of *DFR* expression, while JA and ABA had a synergic effect with sucrose enhancing the induction of the *DFR* gene (Figure 1b).



**Figure 1.** Effect of sucrose and different hormones on anthocyanin biosynthesis.

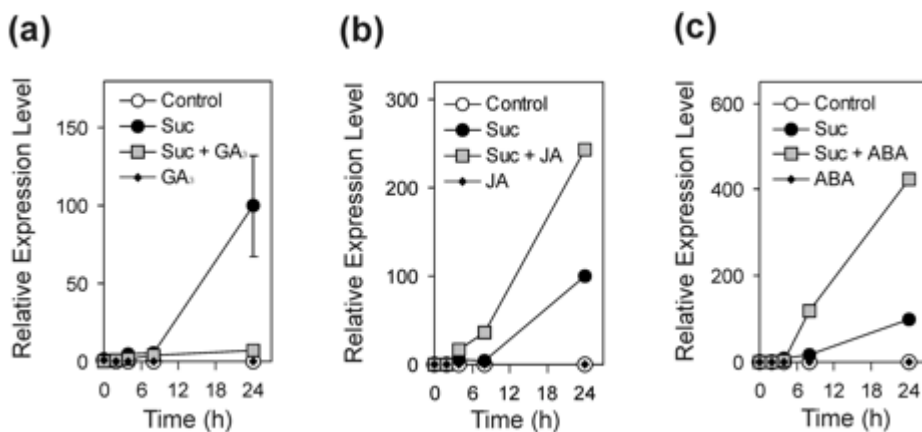
(a) Anthocyanin level was measured in 4-day old seedlings treated with 90mM sucrose and different hormones for 72 hours in the light (data are means of three replicates  $\pm$ SD).

**(b)** *DFR* mRNA level was measured in 4-day old seedlings treated with 90mM sucrose and different hormones for 24 hours. Relative expression level (REL), measured by real-time reverse transcription (RT)-PCR, is shown (REL: 100 = expression data from the sucrose-treated sample). Data are means of three replicates  $\pm$ SD.

**(c)** *DFR* mRNA level was measured in 4-day old seedlings treated with 90mM sucrose for 24 hours, GA<sub>3</sub> (20 $\mu$ M), GA<sub>4</sub> (20 $\mu$ M), and GA<sub>3</sub>+GA<sub>4</sub> (20 $\mu$ M). RNA was extracted, electrophoresed, and northern analysis carried out using gene specific probes. Equal loading was checked by reprobing with an rRNA probe (not shown). A representative experiment is shown.

The repressive effect of gibberellins was further investigated by testing GA<sub>3</sub>, GA<sub>4</sub> and GA<sub>3</sub>+GA<sub>4</sub> to verify whether they are able to repress the sucrose inductive effect on *DFR* mRNA levels (Figure 1c). The results revealed that GA<sub>3</sub> was more effective in counteracting the effect of sucrose than GA<sub>4</sub> and GA<sub>3</sub>+GA<sub>4</sub> (Figure 1c). We thus decided to use GA<sub>3</sub> in subsequent experiments.

To determine the most suitable time point for gene expression analysis, we tested the pattern of *DFR* expression in a time-course experiment. Data showed that a 24h treatment is an appropriate time point to observe the repressive effect of GA<sub>3</sub> and the inductive effect of JA and ABA (Figure 2a-c). Subsequent experiments were therefore performed by treating 4-day-old seedlings for 24h in the absence/presence of exogenous sucrose, GA<sub>3</sub>, JA and ABA: the aim was to identify interactions between these plant hormones and sucrose on most of the genes involved in the anthocyanin biosynthetic pathway.



**Figure 2.** Effect of GA<sub>3</sub>, JA, and ABA on anthocyanin biosynthesis in a time-course experiment. *DFR* mRNA level was measured in 4-day old seedlings treated with 90mM sucrose in the presence and absence of GA<sub>3</sub> **(a)**, JA **(b)** and ABA **(c)**; samples were collected after 2, 4, 9, and 24 hours. Relative expression level (REL), measured by real-time reverse transcription (RT)-PCR, is shown (REL: 100 = expression data from the sucrose-treated sample); data are means of two replicates  $\pm$ SD.

### Gibberellin affects mRNA levels of anthocyanin biosynthetic pathway genes

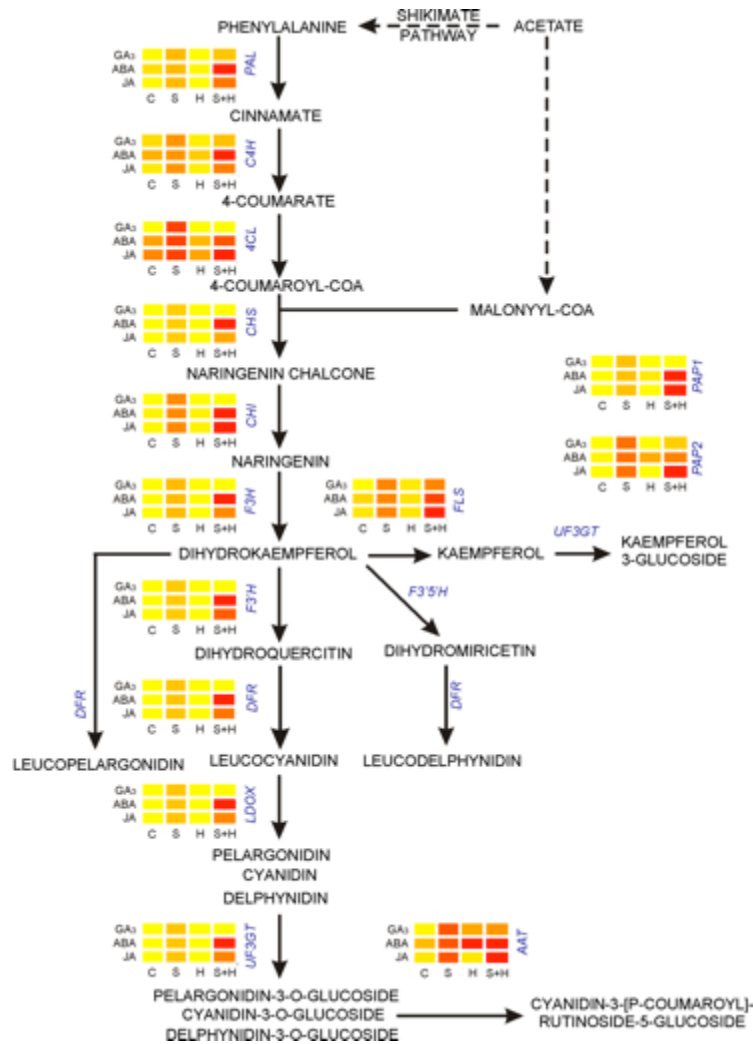
To understand the role of gibberellins on anthocyanin synthesis we studied the interaction between GA<sub>3</sub> and sucrose at a molecular level. This was achieved by analysing the expression pattern of the sucrose-regulated genes along the anthocyanin biosynthetic pathway (Solfanelli et al., 2006). The synthesis precursors of most flavonoids are malonyl-CoA and p-coumaroyl-CoA (Forkmann and Heller, 1999). These two compounds are coupled by chalcone synthase (CHS), which is considered to be the first enzyme involved in flavonoid biosynthesis. The first anthocyanins are instead formed by the action of dihydroflavonol reductase (DFR).

An overview of the inductive effect of sucrose on anthocyanin biosynthetic pathways is reported in Figure 3. Feeding Arabidopsis seedlings with GA<sub>3</sub> had no effect on the genes involved in

the flavonoid/anthocyanin synthesis studied (Figure 3), apart from a moderate inductive effect on the *anthocyanin 5-aromatic acyltransferase (AAT)* mRNA level. GA<sub>3</sub> abolished the positive effects of sucrose on the expression of several anthocyanin biosynthetic genes, including the two transcription factors *MYB75/PAP1* and *MYB90/PAP2* (Figure 3). *PAP1* is known as sucrose-dependent modulators of the anthocyanin pathway (Teng et al., 2005). The repression was evident for those genes coding for enzymes starting from 4-coumarate:CoA ligase (4CL). Besides having anthocyanin as end-product, the pathway can also branch out to other classes of flavonoids, such as flavones (kaempferol, quercetin and myricetin). The step that converts dihydrokaempferol to kaempferol is mediated by flavonol synthase (FLS). In addition, while most plants share the biosynthesis of anthocyanidin 3-glucoside, this product often undergoes further modifications, such as acylation, glycosylation and methylation (Fujiwara et al., 1998). Acylation results in a more stable and bluer anthocyanin. This step is mediated by anthocyanin 5-aromatic acyltransferase (AAT). The sucrose-induction of *FLS* mRNA was barely affected by GA<sub>3</sub>, while a 6-fold *AAT* induction was triggered by GA<sub>3</sub> when compared to the control (Figure 3). Indeed these two genes are not involved in the main pathway of anthocyanin synthesis.

#### **Synergistic effect of jasmonate and sucrose on anthocyanin synthesis genes**

To gain additional clues on the effect of jasmonate on anthocyanin synthesis, we treated 4-day old *Arabidopsis* seedlings with or without jasmonate. Although the positive effect of jasmonate on anthocyanin accumulation is well known (Table 1), we found that jasmonate alone did not affect the biosynthetic pathway in *Arabidopsis* seedlings (Figure 3). In fact, the expression of the transcription factors *PAP1* and *PAP2* was not influenced by jasmonate either (Figure 3). Instead the mRNA accumulation of transcripts related to flavonoid/anthocyanin synthesis, as well as the transcription factors *PAP1* and *PAP2* were induced in the presence of sucrose. Interestingly, when jasmonate was co-supplied with sucrose, it enhanced the expression of most of the genes involved in the pathway, starting at the level of *CHI* and also affecting genes downstream of it (Figure 3). The mRNA levels of *FLS* and *AAT* were induced 1.7 fold in the presence of jasmonate plus sucrose, indicating that the synergistic effect was effective not only for the main pathway leading



**Figure 3.** Effect of sucrose and GA<sub>3</sub>, JA, and ABA on the expression of genes involved in anthocyanin biosynthesis.

Four-day old Arabidopsis seedlings were grown for 24h on a sucrose-free MS medium (Control) or standard medium supplemented with sucrose/hormones alone or fed together. Treatments were as follows: C=control, S=sucrose, H=hormone, S+H=sucrose+hormone. Relative expression level (REL), measured by real-time reverse transcription (RT)-PCR, is shown. Data, averaged transcript level from two biological replicates, were displayed as a heat map. REL is shown as a heat map (REL: 100 = expression data from the sucrose-treated sample). Expression data were visualized using Heatmapper Plus software ([http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools\\_heatmapper\\_plus.cgi](http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi)). The output of the software is shown, with the genes involved in each metabolic step represented by their respective gene symbols. A yellow square (and shades of yellow) indicate a gene whose induction level is relatively low. A red square (and shades of red) indicates a gene whose induction is relatively high within each gene dataset.

to the formation of anthocyanins, but also for the formation of flavones (FLS gene) and the acylation of anthocyanidin 3-glucoside (AAT gene) (Figure 3).

### **Abscisic acid enhances the effects of sucrose on anthocyanin synthesis genes**

Since feeding Arabidopsis seedlings concomitantly with sucrose and ABA resulted in an increased level of *DFR* expression and in a higher level of anthocyanin(s) (Figure 1a), we decided to investigate the role of ABA in more detail. We analyzed the mRNA accumulation of transcripts related to flavonoid/anthocyanin synthesis in ABA enriched media, with or without sucrose. The results indicated that ABA alone did not have effect on any of the genes analysed except for *AAT* and *PAP2* (Figure 3). The induction of *PAP2* in the presence of ABA is in agreement with the results obtained by Tonelli et al. (2007). When ABA was supplied with sucrose, we observed an enhancement in the



sucrose-induction of most of the genes involved in the anthocyanin biosynthesis (Figure 3). Moreover, this enhancement was also detected for the sucrose induction of *PAP1*. The synergic effect of ABA was less evident for those genes (*FLS* and *AAT*) that code for enzymes that represent the side branches of anthocyanin synthesis (Figure 3).

#### **GA synthesis represses the anthocyanin biosynthetic pathway**

We verified whether the expression of anthocyanin genes was affected in *ga1-5*, a mutant impaired in the step catalyzing conversion of geranylgeranyl pyrophosphate (GGPP) to copalyl pyrophosphate (CPP). This GA-deficient mutant germinates without gibberellin treatment (Koornneef and van der Veen, 1980), allowing us to analyze the consequences of a low endogenous gibberellin content on the expression of the genes involved in anthocyanin synthesis, as well as the effects of exogenous GA<sub>3</sub> in both the wild type and mutant. The *ga1-5* microarray dataset (see methods) was analyzed and the results are reported in Figure 4. The expression of the genes coding for enzymes acting downstream of naringenin are expressed at a much higher level in the *ga1-5* mutant, and GA<sub>3</sub> (1 μM) rapidly (3h) lowers the mRNA level. *PAP1* expression is also higher in the *ga1-5* mutant, and GA<sub>3</sub> represses its mRNA level. *PAP2* expression is instead unaffected by the *ga1-5* mutation. These results suggest that a low gibberellin level allows a higher expression of several anthocyanin biosynthetic genes. The expression of *AAT* is instead lower in the *ga1-5* mutant.

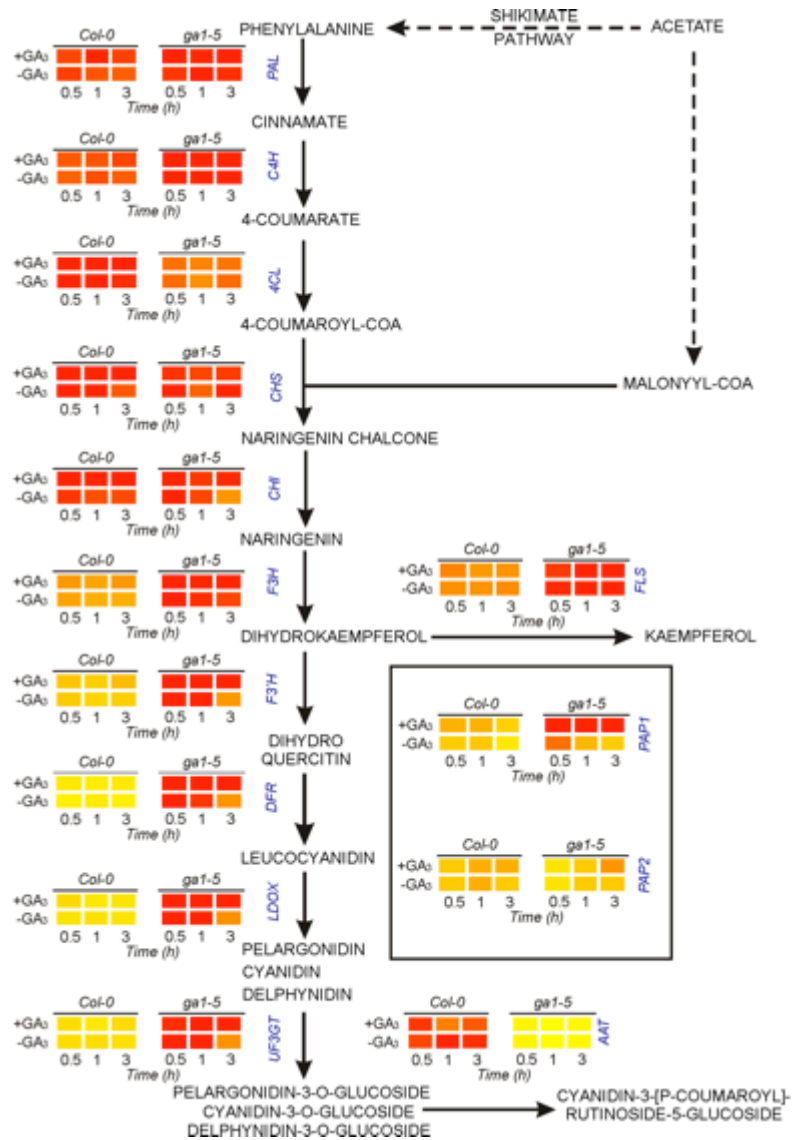
#### **The *gai* mutant is less sensitive to gibberellin-dependent repression of dihydroflavonol reductase**

To gain further insight into the possible interaction between sucrose and gibberellin, we investigated the effect of sucrose in the presence/absence of GA<sub>3</sub> on the gibberellin-insensitive mutant *gai*, a *GAI* gain-of-function mutant impaired in GA(s) signalling (Wilson and Somerville, 1995). Firstly we tested the effect of sucrose supplied with or without GA<sub>3</sub> on the wild-type, Landsberg erecta (Ler) ecotype, which is the genetic background of the *gai* mutant. The results showed that GA<sub>3</sub> was able to counteract the sucrose induction of *DFR* expression in Ler seedlings (Figure 5a), as observed in the Columbia-0 Arabidopsis ecotype (Figure 2a). On the other hand, feeding the *gai* mutant seedlings with sucrose and GA<sub>3</sub> resulted in an expression of *DFR* comparable to that of sucrose alone (Figure 5a). This would seem to prove that *GAI* signalling is involved in the cross-talk between sucrose and gibberellin in wild-type Arabidopsis seedlings. This was replicated and confirmed using leaves from Arabidopsis adult plants (Figure 5b). The induction triggered by sucrose in seedlings (Figure 5a) and leaf-discs (Figure 5b) was much stronger in *gai*, which was less sensitive to the addition of GA<sub>3</sub> (Figure 5a-b).

#### **Sucrose-induction of anthocyanin biosynthesis in ABA deficient and insensitive mutants**

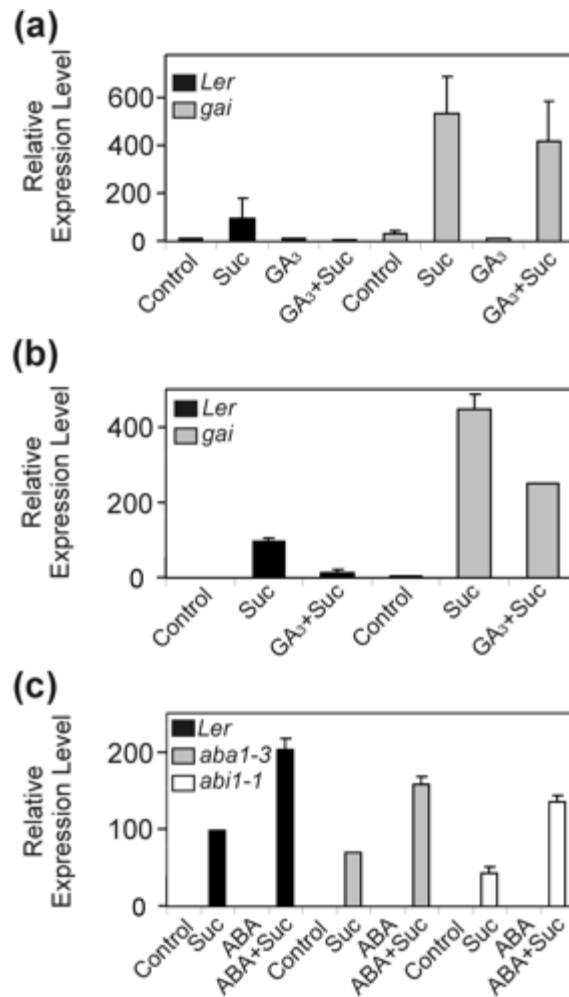
To better understand the role of ABA in anthocyanin accumulation we analyzed the responses of the ABA-deficient mutant (*aba1-3*), impaired in the epoxidation of zeaxanthin and antheraxanthin to violaxanthin (Rock and Zeevaart, 1991), together with the ABA-insensitive (*abi1-1*; Koornneef et al., 1984) mutant to ABA, with and without sucrose. The expression levels of *DFR* in *aba1-3*, *abi1-1*, and in the wild type Landsberg erecta was analyzed after sucrose, and after sucrose with ABA treatments (Figure 5c). Assuming that the inductive effect of sucrose was ABA-mediated, a low level of *DFR* expression would have been expected in the presence of sucrose in the *aba1-3* mutant. Our results showed that *DFR* expression level in sucrose-treated *aba1-3* was only slightly lower than that of the wild type, suggesting that the sucrose-driven induction of the anthocyanin pathway is not strictly dependent on the synthesis of ABA. To clarify the possible interaction between abscisic acid and sucrose, we investigated the effect of this sugar in the presence/absence of ABA on the abscisic acid-

insensitive mutant (*abi1-1*). Our results showed that the synergistic effect of ABA and sucrose was retained in the *abi1-1*, indicating the *ABI1* is not required for the effects of abscisic acid on the sucrose-induction anthocyanin pathway (Figure 5c).



**Figure 4.** Effect of gibberellin on the *ga1-5* mutant.

The AtGenExpress dataset TAIR-ME00343 (GA<sub>3</sub> time course in *Col-0* and *ga1-5* mutant seedlings) was used. The dataset was obtained using seven-day old Arabidopsis seedlings grown for 0.5, 1, 3h on MS medium (-GA<sub>3</sub>) or medium supplemented with GA<sub>3</sub> (1 μM) under continuous light at 23°C. Expression data were visualized using Heatmapper Plus software ([http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools\\_heatmapper\\_plus.cgi](http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi)). The output of the software is shown, with the genes involved in each metabolic step represented by their respective gene symbols. A yellow square (and shades of yellow) indicate a gene whose induction level is relatively low. A red square (and shades of red) indicates a gene whose induction is relatively high within each gene dataset.



**Figure 5.** Effect of sucrose, GA<sub>3</sub>, and ABA in Arabidopsis mutants.

**(a)** *DFR* mRNA level in seedlings (4-d old) of the wild-type (*Ler*) and *gai* mutant.

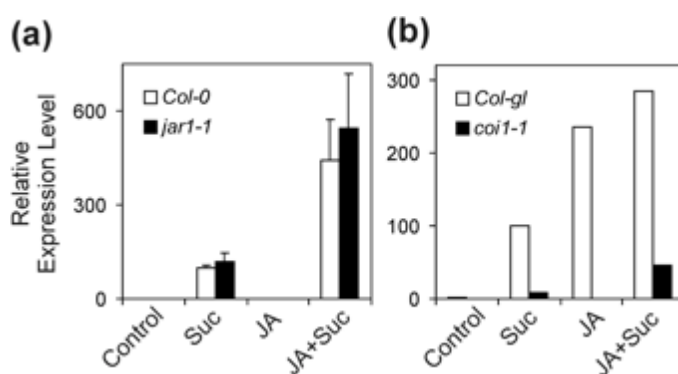
**(b)** *DFR* mRNA level in leaf discs of the wild-type (*Ler*) and *gai* mutant.

**(c)** *DFR* mRNA level in seedlings (4-d old) of the wild-type (*Ler*) and *aba1-3* and *abi1-1* mutants.

Experimental conditions: (a, c) four-day old Arabidopsis seedlings were grown for 24h on a sucrose-free MS medium (Control) or MS medium supplemented with sucrose/hormones alone or fed together; (b) Leaf discs were excised from Arabidopsis plants (rosette stage) and treated for 24h on a sucrose-free MS medium (Control) or MS medium supplemented with sucrose or GA<sub>3</sub> with sucrose. Relative expression level (REL), measured by real-time reverse transcription (RT)-PCR, is shown. (REL: 100 = expression data from the sucrose-treated *Ler* sample). The results are mean ± SD (n=2).

### ***COI1* is involved in the sucrose-dependent induction of dihydroflavonol reductase**

Mutants defective in JA-signalling include *coronatine insensitive 1 (coi1)* and *jasmonate-insensitive 1 (jar1)* mutant (Feys et al., 1994; Staswick et al., 1992). The response of Arabidopsis to JA requires the *COI1* gene, since *coi1* mutants fail to express JA-induced genes (Feys et al., 1994; Benedetti et al., 1995; Ellis and Turner 2002). The induction of genes involved in anthocyanin production following treatment with JA in the presence of sucrose is defective in *coi1* mutants (Devoto et al., 2005; Kim et al., 2006; Chen et al., 2007). We therefore investigated the role of *COI1* and *JAR1* in the sucrose-dependent induction. Our results showed that the induction triggered by sucrose is retained in *jar1-1* (Figure 6a), and JA is still able to enhance the sucrose-dependent expression of *DFR*, in agreement with Chen et al., (2007). The need to select homozygous *coi1-1* plants by germination on a jasmonate-containing medium hampers the use of seedlings as experimental material. We therefore selected homozygous *coi1-1* plants, and used leaf strips in the experiments. The expression of *DFR* in leaf strips is positively affected by sucrose, JA+Suc and, interestingly, also by jasmonate, a likely consequence of the higher sugar content of photosynthetic tissues when compared to germinating seedlings. The effect of jasmonate is, as expected, strongly reduced in *coi1-1*. Interestingly, not only JA effects were negligible in *coi1-1*, but also the response to sucrose was lost in the mutant, suggesting that *COI1* plays a role in the sucrose-dependent signalling pathway by modulating anthocyanin biosynthesis (Figure 6b).



**Figure 6.** Role of *JAR1* and *COI1* in the sucrose-induction of *DFR*.

**(a)** *DFR* mRNA level in four-day old *Col-0* and in the *jar1-1* Arabidopsis seedlings grown for 24h on a sucrose-free MS medium (Control) or MS medium supplemented with sucrose.

**(b)** *DFR* mRNA level in *Col-gl* and *coi1-1* plants. Leaf strips from *Col-gl* and *coi1-1* plants were used in this experiment. See methods for the procedure used to identify homozygous *coi1-1* mutants. Relative expression level (REL), measured by real-time reverse transcription (RT)-PCR, is shown. (REL: 100 = expression data from the sucrose-treated *Col* samples).

## Discussion

Anthocyanin biosynthesis is regulated by a complex interaction between internal and external stimuli such as temperature, light, carbohydrates, water stress and plant hormones.

The involvement of plant hormones on anthocyanin accumulation is an intriguing field of research, but the literature on this subject is still controversial (see Table 1). Taking advantage of a model plant system such as *Arabidopsis*, we were interested in investigating the possible interactions between sugars and hormones in the regulation of anthocyanin biosynthesis. Sucrose affects genes involved in anthocyanin biosynthesis and the effect is sucrose-specific for *DFR* and genes downstream of it (Solfanelli et al., 2005). The effects of sucrose on genes upstream of *DFR* are therefore not necessarily related to sucrose acting as a specific signalling pathway, but may be linked to sensing of exos arising from sucrose degradation.

None of the plant hormones used were able to affect significantly the expression of the anthocyanin biosynthetic genes unless sucrose was fed concomitantly (Figure 1a-b). Our results indicated that gibberellins, jasmonate and abscisic acid, but not auxin, ethylene and cytokinins, may interact or cross-talk with sucrose to form a complex web of overlapping signalling pathways that coordinate anthocyanin accumulation.

Gibberellins counteracted the sucrose induction of anthocyanin biosynthesis (Figure 1a-b; Figure 2a; Figure 3). The sucrose-induction of *PAP1* and *PAP2* (Borevits et al., 2000), known as sucrose-dependent modulators of the anthocyanin pathway (Teng et al., 2005; Solfanelli et al., 2006), were repressed with the addition of gibberellic acid (Figure 3). Genes acting downstream of naringenin are up regulated in the *ga1-5* mutant, indicating that a low endogenous gibberellin level favours the expression of the anthocyanin pathway genes (Figure 4). Interestingly, the expression of *AAT* is, instead, lower in the *ga1-5* mutant (Figure 4), in agreement with the positive effects of  $GA_3$  on the expression of this gene (Figure 3). The use of *gai* mutant seedlings (Peng et al., 1997) revealed that *GAI* is involved in the cross-talk between sucrose and gibberellins (Figure 5a-b). Jiang et al. (2007) detected a low level *F3'H*, *LDOX*, and *UF3GT* expression in leaves of a loss-of-function *GAI* mutant, suggesting that *GAI*, a DELLA protein, is required for the expression of these genes. Interestingly, the expression level of *DFR* was much higher in seedlings and leaves of the *gai* mutant (Figure 5a-b), suggesting that gibberellins modulate the anthocyanin biosynthetic pathway through the activity of DELLA proteins. This is in agreement with Jiang et al. (2007) who demonstrated that the phosphate-starvation-dependent expression of some genes involved in the anthocyanin pathway requires the activity of DELLA proteins.

The jasmonate family of signalling molecules regulates responses to many biotic and abiotic stresses (Turner et al., 2002; Devoto and Turner, 2003; Balbi and Devoto, 2007). Moreover, JAs may also stimulate anthocyanin accumulation in many plant systems (Table 1). JA enhanced the sucrose-induction of the entire biosynthetic pathway, including the two transcription factors *PAP1* and *PAP2* (Figure 3). The synergistic effect was present at mRNA level (Figure 2b; Figure 3) and correlates with anthocyanin accumulation (Figure 1a). In agreement with our results, feeding corn (*Zea mays* L.) leaves with JA and sucrose resulted in enhanced anthocyanin content (Kim et al., 2006). In *Arabidopsis*, the ability of jasmonate to induce several anthocyanin genes has been reported elsewhere (Devoto et al., 2005; Chen et al., 2007). In our study, however, JA applied to *Arabidopsis* in the absence of sucrose had no effect on the expression of gene coding for enzymes involved in the anthocyanin biosynthetic pathway. This discrepancy is easily explained, since sucrose was probably present in the experiments performed by Devoto et al. (2005) and Chen et al. (2007) who grew their seedlings in Murashige-Skoog medium, which contains enough sucrose (58 mM) to induce the anthocyanin genes (Solfanelli et al., 2006). The JA effect observed is thus likely to be an enhancing effect of JA on the sucrose-dependent pathway or, alternatively, sucrose represents a pre-requisite

for the action of jasmonate. It was recently shown that the jasmonate-insensitive *coi1-2* mutant is not JA responsive in terms of *DFR* expression and that *COI1*, but not *JAR1* genes, is required for JA-mediated anthocyanin accumulation (Chen et al., 2007). Our results indicate that *COI1* is involved in the sucrose-dependent signalling pathway (Figure 6), suggesting a convergence of the sucrose and jasmonate signalling pathways.

In several plant systems ABA induced anthocyanin accumulation, whereas in others it had a repressive role (Table 1). ABA co-supplied with sucrose showed a synergistic effect on anthocyanin accumulation and gene expression. In our experiments ABA alone had an inductive effect only on *AAT* and *PAP2* genes. It was recently reported that *PAP2* (*AtMYB90*) is up-regulated in response to drought, salt and ABA treatments (Tonelli et al., 2007). The anthocyanin pathway in maize was blocked in the *viviparous-1* (*vp1*) mutant, an abscisic acid insensitive mutant (McCarty et al., 1989). The block in anthocyanin synthesis in the *vp1* mutant is associated with the failure to express the *C1* gene, an MYB transcription factor similar to Arabidopsis *PAP1* and *PAP2*, during the seed maturation of maize (McCarty et al., 1989). In addition, it was demonstrated that the activity of *C1* promoters was not only regulated by *Vp1* but also by ABA (Hattori et al., 1992). This evidence led us to investigate the role of ABA in the presence and absence of sucrose in mutants altered in their ability to produce ABA or transduce the ABA signals (Figure 5c). Our results showed that *DFR* mRNA levels in *aba1-3* and in *abi1-1* mutants were similar to those of the wild type, suggesting that ABA was not strictly required for sucrose-dependent anthocyanin induction (Figure 5c).

Our results suggest a possible convergence of the sucrose and hormone signalling pathways in the regulation of the anthocyanin biosynthetic pathway. It is tempting to speculate about the possible involvement of the ubiquitination/proteasome pathway for protein degradation as a player in both phytohormone and sugar responses (Ellis et al., 2002). The Arabidopsis *ASK1* gene, which encodes a subunit of a SCF ubiquitin ligase, is involved in the response to jasmonate (Santner and Estelle, 2007) and interacts with the Arabidopsis *KIN10* and *KIN11*, two SNF1-related protein kinases (SnRK) (Farrás et al., 2001). Interestingly, sucrose represses *KIN10*, a SnRK1 that, when over-expressed, leads to repression of *PAP1* (Baena-Gonzalez et al., 2007). SnRKs are important elements in sugar responses (Halford et al., 2003; Rolland et al., 2006), and these results suggest a possible SnRK-ASK1 function in both jasmonate and sugar responses. Sucrose-dependent interaction of SnRK1 proteins may lead to a faster degradation of JAZ protein by the SCF<sup>COI1</sup> complex (Santner and Estelle, 2007), triggering de-repression of the MYC2/JAZ complex which in turn leads to the expression of JA-regulated anthocyanin genes. In this scenario, possibly through SnRK activity, sucrose acts as an activator of SCF complexes and would therefore represent a prerequisite for JA action. Since gibberellin signalling depends on SCF<sup>SLY1</sup> activity, which causes the destruction of the DELLA proteins that repress the action of GAs, the negative effects of GAs on the anthocyanin biosynthetic pathway can also be explained by hypothesizing a sucrose-enhanced SCF<sup>SLY1</sup> activity. This activity, in the presence of GAs, induces the transcription of GA-dependent genes encoding repressor(s) of the anthocyanin genes. The *gai*-encoded DELLA protein cannot be degraded by the SCF<sup>SLY1</sup> complex, resulting in the constitutive repression of the GA-dependent signalling pathway and, consequently, a higher expression of the *DFR* gene can be observed (Figure 5a-b). GA-treatments or mutants with reduced DELLA function result in an inability to induce some anthocyanin genes (Jiang et al., 2007), supporting the requirement of repressed GA-signalling as a pre-requisite for the induction of the GA-biosynthesis pathway. In this framework, the ABA effect can also be seen as part of a mechanism with DELLA proteins playing a central role. In fact it has been demonstrated that ABA increases the stability of RGA and blocks its GA-induced degradation (reviewed by Weiss and Ori, 2007). ABA may therefore act as a repressor of the GA-pathway and de-repress the synthesis of anthocyanin genes. Further research is thus required to elucidate these interacting regulative networks.

Overall, our results indicated that a cross-talk between sucrose and hormones controls anthocyanin biosynthesis in *Arabidopsis thaliana*. The ability of plant hormones to modulate the anthocyanin biosynthetic pathway is highly dependent on the presence of sucrose acting as a consensus-signalling molecule. It thus appears that an adequate metabolic status, signalled by the sugar plant level, is a pre-requisite for the action of plant hormones to regulate anthocyanin accumulation.

## Materials and methods

### Plant and growth condition

*Arabidopsis thaliana*, ecotype Columbia (Col-0; if not otherwise specified) seeds were sterilized for 7 min in 1.7% (v/v) bleach solution, incubated over night in 4% PPM (Plant Preservative Mixture, Plant Cell Technology, Washington DC, USA) in a full strength sterilized Murashige-Skoog (MS) salt solution with gentle shaking. Subsequently the seeds were rinsed in abundant sterile water and transferred into 2.5 ml liquid growing media (MS half strength solution +/- sugars) with 0.05% PPM in 6-well plates. The plates were incubated in the dark at 4°C for two days and finally transferred to continuous light (90µm photons/ m<sup>-2</sup>) with gentle swirling for four days in a plant growth chamber at 22°C. Treatments were performed by adding a sugar/hormone solution to selected wells and water to the control wells. Sugar and hormones were used at the following concentrations, unless differently indicated: sucrose 90mM, GAs 20µM; ABA 5µM; BA 10µM; JA 45µM; ACC 50µM; 2,4D 1µM. For the *coi1-1* experiment, seeds were germinated on jasmonate (30 µM) to identify *coi1-1* homozygous plants (Feys et al., 1994); wild-type (Col-gl) seeds were germinated on JA-free medium. Fourteen-day old wild-type (Col-gl) and *coi1-1* *Arabidopsis* plants were collected and transferred in pots containing a peat-based substrate. Plants were grown in a growth chamber for 20 days (22°C, 110µm photons/ m<sup>-2</sup>, 11/13h light/night photoperiod). Leaves were collected, cut in 0.5 cm-wide strips and treated for 24h on a sucrose-free MS medium (Control) or MS medium supplemented with sucrose, JA, JA+Suc.

### Anthocyanin quantification

*Arabidopsis* seedling extraction was performed as described by Ronchi et al., (1997) with minor modifications. In brief, seedlings were ground in one volume HCl 1% (v/v) in methanol with the addition of 2/3 volume of distilled water. Extracts were recovered and one volume of chloroform was added to remove chlorophylls by mixing and centrifugation (1 min at 14000 g). The anthocyanin contained in the aqueous phase were recovered and absorbance was determined spectrophotometrically (A<sub>535</sub> nm). Mean values were obtained from three independent replicates.

### RNA Isolation and gel blots

RNA extraction was performed by using the aurintricarboxylic acid method as previously described (Perata et al., 1997). The amount of total RNA loaded per lane for electrophoresis was 20 µg. RNA was electrophoresed on 1% (w/v) agarose glyoxal gels, and blotted on a nylon membrane (BrightStar-Plus®, Ambion, Austin, Texas USA) using the procedure recommended by the manufacturer. Membranes were prehybridized and hybridized using a NorthernMax-Gly® kit (Ambion, Austin, Texas USA). Radiolabeled probes were prepared from gel-purified cDNAs by random primer labeling (Takara Chemicals, Shiga, Japan) with [ $\alpha^{32}$ P]-dCTP. Equal loading was checked by reprobing with an rRNA cDNA probe (data not shown). RNA blots were scanned using a Cyclone Phosphoimager (Packard Bioscience, Perkin Elmer, Foster City, CA, USA). The mRNA level was quantified using Optiquant software (Packard Bioscience, Perkin Elmer, Foster City, CA, USA).



### Expression analysis

The total RNA, extracted using the RNAqueous kit (Ambion, Austin, Texas, USA) according to the manufacturer's instructions, was subjected to DNase treatment using a TURBO DNA free kit (Ambion). Two micrograms of each sample were reverse transcribed into cDNA with a "High capacity cDNA archive kit" (Applied Biosystems, Foster City, CA, USA). RealTime PCR amplification was carried out using an ABI Prism® 7000 Sequence Detection System (Applied Biosystems, Foster City, USA), with primers described in Supplemental Table 2. *Ubiquitin10* (*UBQ10*) was used as an endogenous control. Specific Taqman probes for each gene were used. Probe sequences are reported in Table 2. PCR reactions were carried out using 50 ng of cDNA and "TaqMan Universal PCR Master Mix" (Applied Biosystems) following the manufacturer's protocol. Relative quantitation of each single gene expression was performed using the comparative C<sub>T</sub> method as described in the ABI PRISM 7700 Sequence Detection System User Bulletin #2 (Applied Biosystems). In order to allow an easier comparison of the effects of hormones on the induction of genes, we arbitrary set to "100" the expression level of the sucrose-treated samples, a treatment in common with all the experimental conditions used. The relative expression levels were represented as heatmaps in Figure 3, using Heatmapper Plus software, a general tool for applying a third dimension of information via colour-coding to a 2-D table. ([http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools\\_heatmapper\\_plus.cgi](http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi)). The AtGenExpress dataset TAIR-ME00343 (GA<sub>3</sub> time course in wildtype and *ga1-5* mutant seedlings) was used to evaluate the effects of GA-deficiency of the expression of the anthocyanin-related genes. Data were visualized using the using Heatmapper Plus software.

**Table 2.** List of primers and Taqman probes used in Real Time PCR analysis.

<i>Gene</i>	<i>Description</i>	<i>5'-3' sequence</i>
<b>At1g56650 (PAP1)</b>	primer forward	CCGCAAATGACGTCAAGAATTACTG
	primer reverse	GGAATGGGCGTAATGTCTCTCTTTT
	TaqMan probe	ACACGGTTCATGTTTCTTAC
<b>At1g51680 PAP2</b>	primer forward	GTGAAAATAGTATCACATGTAACAAAGATGATGAG
	primer reverse	CCCCAGTAAATTCTCCAACCACATA
	TaqMan probe	AAAGATGATTTTTGTGAATAATC
<b>At2g37040 PAL</b>	primer forward	CCAAAAACGGTGTGCGCACT
	primer reverse	GCTCCGAATATTCCGGCGTTAA
	TaqMan probe	TCAGAAGGAAGTTATTAGATTCC
<b>At2g30490 C4H</b>	primer forward	GGAGAAATCAACGAGGACAATGTTC
	primer reverse	CCACTCGATAGACCACAATGTTGT
	TaqMan probe	CAATCGCGGGCAGATT
<b>At3g48990 4CL</b>	primer forward	GTGGGTCGGATCAAAGAGCTT
	primer reverse	AGAGTACTGCATCCACTTCAATTGG
	TaqMan probe	CTCACCTCCACGGTTAAT
<b>At5g13930 CHS</b>	primer forward	CCGACCTCAAGGAGAAGTTCAAG
	primer reverse	GCATGTGACGTTTCCGAATTGT
	TaqMan probe	CATGTGCGACAAGTCG
<b>At5g05270 CHI</b>	primer forward	GAATCTATCCCCTTCTCCGTGAA
	primer reverse	GGCAGTTTCATTGTACCTTGATAA
	TaqMan probe	ACGCACCGGTGACTAT
<b>At3g51240 F3H</b>	primer forward	AGGAGCGTTTGTGTCATCTC
	primer reverse	GCATTCTTGAACCTCCCATTGC
	TaqMan probe	ACGGCCATTTTTG
<b>At5g63590 FLS</b>	primer forward	CTTCCGGGATCATCGTCATCATC
	primer reverse	GCCCTATGCTCCACACTTATACT
	TaqMan probe	CATTGCTCATTCTCAAGAAT
<b>At5g07990 F3'H</b>	primer forward	CAGACATCGCTCAGCTTCCTTA
	primer reverse	GGTGGATGAAGCCTGAAATTCTCTT
	TaqMan probe	CCTCAGGCGGTTATC
<b>At5g42800 DFR</b>	primer forward	CCTTATCACCGGCTCTCT
	primer reverse	TGTCCTTGTCTTATGATCGAGTAATGC
	TaqMan probe	CCTCGTCCGAGTGATAG
<b>At4g22870 LDOX</b>	primer forward	TCAATTTGGCCTAAGACACCAAGT
	primer reverse	TCGCTAGCAAACGAAGACACTT
	TaqMan probe	ACTCGTTGCTTCTATGTAATC
<b>At5g54060 UF3GT</b>	primer forward	CAACTGGTTTTCCGTTTCTGGTT
	primer reverse	GCTTCCTCGACGGTTGATACAC
	TaqMan probe	CCGAAGGAGGCTTAATG
<b>At5g61160 AAT</b>	primer forward	TGTTTCTACCTTCGTGGTACTCTA
	primer reverse	TGTGGTATTGGTTGTGTACTTGAG
	TaqMan probe	ACGATTTCCAAATGAAAGC

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