



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

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## Summary

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• Anthocyanins are secondary metabolites, which play an important role in the physiology of plants. Both sucrose and hormones regulate anthocyanin synthesis. Here, the interplay between sucrose and plant hormones was investigated in the expression of sucrose-regulated genes 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 analysed by real-time reverse transcriptase polymerase chain reaction (RT-PCR) in *Arabidopsis* seedlings treated with sucrose and plant hormones.

• Sucrose-induction of the anthocyanin synthesis pathway was repressed by the addition of gibberellic acid (GA) whereas jasmonate (JA) and abscisic acid (ABA) had a synergic effect with sucrose. The *gai* mutant was less sensitive to GA-dependent repression of *dihydroflavonol reductase*. This would seem to prove that *GAI* signalling is involved in the crosstalk between sucrose and GA in wild-type *Arabidopsis* seedlings. Conversely, the inductive effect of sucrose was not strictly ABA mediated. Sucrose induction of anthocyanin genes required the *CO1* gene, but not *JAR1*, which suggests a possible convergence of the jasmonate- and sucrose-signalling pathways.

• The results suggest the existence of a crosstalk between the sucrose and hormone signalling pathways in the regulation of the anthocyanin biosynthetic pathway.

**Key words:** abscisic acid (ABA), anthocyanin, *Arabidopsis*, gibberellin (GA), jasmonate (JA), sucrose, sugar sensing.

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## Introduction

Flavonoids represent a large class of secondary plant metabolites, of which anthocyanins are the most conspicuous, owing to the wide range of chemical structures derived from their synthesis. In addition to 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 ultraviolet irradiation damage (Winkel-Shirley, 2001). Anthocyanins are considered as antioxidant molecules (Gould *et al.*, 2002; Gould, 2004) 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 characterized in several plant species including *Arabidopsis* (Shirley *et al.*, 1995; Bharti & Khurana, 1997). The basal level

\*These authors contributed equally to this work.

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

	Enhancing effects	Inhibiting effects
Gibberellins	Petunia flowers (Weiss <i>et al.</i> , 1995; Moalem-Beno <i>et al.</i> , 1997); Periwinkle flowers (Ohlsson & Berglund, 2001); <i>Hyacinthus</i> sepals (Hosokawa, 1999)	Strawberry fruit (Martinez <i>et al.</i> , 1996); Maize leaves (Kim <i>et al.</i> , 2006); Carrot cell cultures (Ilan & Dougall, 1992, 1994); Low phosphate grown <i>Arabidopsis</i> (Jiang <i>et al.</i> , 2007)
Cytokinins	Maize seedlings (Piazza <i>et al.</i> , 2002); <i>Arabidopsis</i> seedlings (Deikman & Hammer, 1995; Laxmi <i>et al.</i> , 2006); Strawberry cell cultures (Mori <i>et al.</i> , 1994)	Maize leaves (Kim <i>et al.</i> , 2006); <i>Arabidopsis</i> seedlings (Wade <i>et al.</i> , 2003)
Ethylene	Maize leaves (Kim <i>et al.</i> , 2006); Grape berry skin (El-Kereamy <i>et al.</i> , 2003)	
2,4D (2,4-dichlorophenoxyacetic acid)	Strawberry cell cultures, high auxin treatment (Mori <i>et al.</i> , 1994)	Strawberry cell cultures (Mori <i>et al.</i> , 1994); Grape berry skin (Ban <i>et al.</i> , 2003; Jeong <i>et al.</i> , 2004)
Abscisic acid	Maize leaves (Kim <i>et al.</i> , 2006); Grape berry skin (Ban <i>et al.</i> , 2003; Jeong <i>et al.</i> , 2004; Mori <i>et al.</i> , 2005); Maize kernels (Hattori <i>et al.</i> , 1992; Paek <i>et al.</i> , 1997)	Grape berry skin (Han <i>et al.</i> , 1996); Petunia flowers (Weiss <i>et al.</i> , 1995)
Jasmonate	Strawberry fruit (Ayala-Zavala <i>et al.</i> , 2005); Tulip bulbs (Saniewski <i>et al.</i> , 1998b, 2004); <i>Arabidopsis</i> seedlings (Devoto <i>et al.</i> , 2005; Chen <i>et al.</i> , 2007); Maize leaves (Kim <i>et al.</i> , 2006); Soybean plants (Franceschi & Grimes, 1991); Carrot cell cultures (Sudha & Ravishankar, 2003); Kalanchoe cell cultures (Saniewski <i>et al.</i> , 2003); Potato cell cultures (Plata <i>et al.</i> , 2003); Apple fruit (Rudell <i>et al.</i> , 2002); Peach shoots (Saniewski <i>et al.</i> , 1998a); Petunia flowers (Tamari <i>et al.</i> , 1995)	

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 (JA), abscisic acid (ABA), stress-inducible, and pathogenesis-related genes are also coregulated 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). More-

over, 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 crosstalk to form a complex network of overlapping signalling, which coordinate overall plant growth and development (Loreti *et al.*, 2000; Smeekens, 2000; Leon & Sheen, 2003; Rolland *et al.*, 2006). The influence of exogenous plant growth regulators on anthocyanin accumulation is, however, not fully understood. Jasmonate 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). In addition, ethylene appears to have a univocally positive effect on anthocyanin accumulation, whereas the results are contradictory for gibberellins (GAs), 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 (2,4-dichlorophenoxyacetic acid, 2,4D), and cytokinins is contradictory (Table 1), although these results can be explained by taking into account the different plant species/organs studied. 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 paper, we described the sucrose-specific induction of anthocyanin biosynthesis in *Arabidopsis* seedlings (Solfanelli *et al.*, 2006). In this study, we investigated the interaction between sucrose and plant hormones. The results showed that, while gibberellic acid (GA<sub>3</sub>) inhibited the sucrose induction of most of the genes involved in the anthocyanin pathway, JA and ABA had a synergic effect with sucrose, enhancing the induction of these genes.

## Materials and Methods

### Plant and growth conditions

*Arabidopsis thaliana* (L.) Heynh, 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 excess sterile water and transferred into 2.5 ml liquid growing media (MS half-strength solution ± sugars) with 0.05% PPM in six-well plates. The plates were incubated in the dark at 4°C for 2 d and finally transferred to continuous light (90 μm photons m<sup>-2</sup>) with gentle swirling for 4 d 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 90 mM; GAs 20 μM; ABA 5 μM; benzyl adenine (BA) 10 μM; Methyl-JA (JA) 45 μM; 1-aminocyclopropane-1-carboxylic acid (ACC) 50 μM; 2,4D 1 μM. Mutants used include the GA insensitive *gai* mutant (background: *Ler*), the ABA mutants *aba1-3* and *abi1-1* (ABA deficient and insensitive, respectively; background: *Ler*), the JA-insensitive *jar1-1* and *coi1-1* (background: *Col-0* and *Col-gl* respectively). For the *coi1-1* experiment, seeds were germinated on JA (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 d (22°C, 110 μm photons m<sup>-2</sup>, 11 h : 13 h light : dark photoperiod). Leaves were collected, cut into 0.5-cm wide strips and treated for 24 h on a sucrose-free MS medium (control) or MS medium supplemented with sucrose and JA (JA + Suc).

### Anthocyanin quantification

*Arabidopsis* seedling extraction was performed as described by Ronchi *et al.* (1997) with minor modifications. Briefly,

seedlings were ground in one volume of 1% HCl (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 14 000 g). The anthocyanin contained in the aqueous phase was recovered and absorbance determined spectrophotometrically (A<sub>535</sub> nm). Mean values were obtained from three independent replicates.

### RNA isolation and gel blots

The 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, TX, USA) using the procedure recommended by the manufacturer. Membranes were prehybridized and hybridized using a NorthernMax-Gly kit (Ambion). Radiolabelled probes were prepared from gel-purified cDNAs by random primer labelling (Takara Chemicals, Shiga, Japan) with [α<sup>32</sup>P]-dCTP. Equal loading was checked by reprobating with an rRNA cDNA probe. RNA blots were scanned using a Cyclone Phosphorimager (Packard Bioscience, Perkin Elmer, Foster City, CA, USA). The mRNA level was quantified using Optiquant software (Packard Bioscience).

### Expression analysis

The total RNA, extracted using the RNAqueous kit (Ambion) 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). Real-time polymerase chain reaction (PCR) amplification was carried out using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA), with primers described in the Supporting Information, Table S1. Ubiquitin10 (*UBQ10*) was used as an endogenous control. Specific Taqman probes for each gene were used. Probe sequences are reported in the Supporting Information, Table S1. PCR reactions were carried out using 50 ng of cDNA and 'TaqMan Universal PCR Master Mix' (Applied Biosystems) following the manufacturer's protocol. Relative quantification 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 Fig. 3, using HEATMAPPER PLUS software, a general tool for applying a third dimension of information via colour-coding to a two-dimensional

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)). HEATMAPPER PLUS contrast was set to the maximal value in the dataset. The actual numerical data are reported in the Supporting Information, Table S2.

The AtGenExpress dataset (Winter *et al.*, 2007) TAIR-ME00343 ( $GA_3$  time course in wild-type and *gal-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.

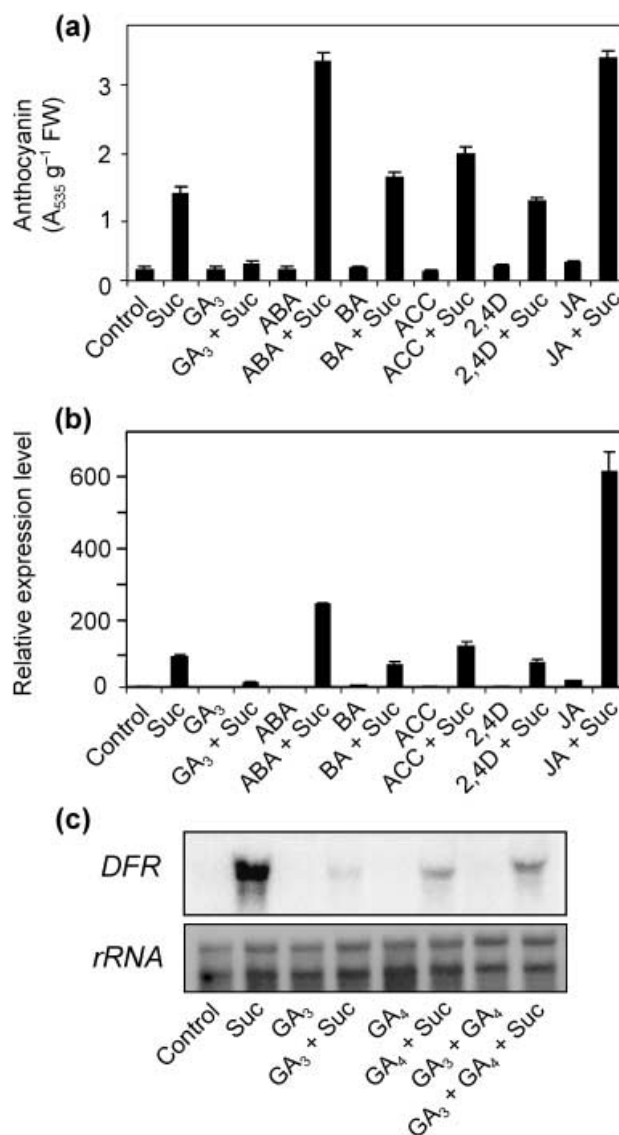
## Results

### Effects of different hormones on anthocyanin synthesis

Sucrose increases anthocyanin content in *Arabidopsis* seedlings (Teng *et al.*, 2005; Solfanelli *et al.*, 2006). In order to gain additional clues regarding sucrose regulation, the effect of hormones on the sucrose-induction of anthocyanin accumulation in *Arabidopsis* seedlings were tested. Hormones alone were unable to affect anthocyanin levels under our experimental conditions (Fig. 1a). Treatments with BA, 2,4D and ACC, in combination with sucrose, did not significantly influence anthocyanin contents with respect to the sucrose-alone treatment (Fig. 1a). Deikman & 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 (Fig. 1a). Interestingly, when  $GA_3$  was supplied together with sucrose, an inhibiting effect on the sucrose-induction of anthocyanins was observed (Fig. 1a). By contrast, the addition of JA and ABA to sucrose was very effective in enhancing anthocyanin accumulation (Fig. 1a). The mRNA level of dihydroflavonol reductase (*DFR*), a sucrose-induced gene involved in anthocyanin synthesis was also measured (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 (Fig. 1b).

The repressive effect of GAs was further investigated by testing  $GA_3$ ,  $GA_4$  and  $GA_3 + GA_4$  to verify whether they are able to repress the sucrose inductive effect on *DFR* mRNA concentrations (Fig. 1c). The results revealed that  $GA_3$  was more effective in counteracting the effect of sucrose than  $GA_4$  and  $GA_3 + GA_4$  (Fig. 1c); therefore  $GA_3$  was used in subsequent experiments.

To determine the most suitable time-point for gene expression analysis, the pattern of *DFR* expression was tested in a time-course experiment. Data showed that a 24-h treatment is appropriate to observe the repressive effect of  $GA_3$  and the inductive effect of JA and ABA (Fig. 2). Subsequent experiments were therefore performed by treating 4-d-old seedlings for 24 h in the absence/presence of exogenous sucrose,  $GA_3$ , 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.



**Fig. 1** Effect of sucrose and different hormones on anthocyanin biosynthesis. (a) Anthocyanin level was measured in 4-d-old *Arabidopsis* seedlings treated with 90 mM sucrose and different hormones for 72 h in the light (data are means of three replicates  $\pm$  SD). (b) *DFR* mRNA level was measured in 4-d-old seedlings treated with 90 mM sucrose and different hormones for 24 h. Relative expression level (REL), measured by real-time reverse transcription polymerase chain reaction (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-d-old seedlings treated with 90 mM sucrose for 24 h,  $GA_3$  (20  $\mu$ M),  $GA_4$  (20  $\mu$ M), and  $GA_3 + GA_4$  (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. A representative experiment is shown. Suc, sucrose; GA, gibberellin; ABA, abscisic acid; BA, benzyl adenine; ACC, 1-aminocyclopropane-1-carboxylic acid; 2,4-dichlorophenoxyacetic acid (auxin); JA, jasmonate.



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

To understand the role of GAs 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 & 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 Fig. 3. Feeding *Arabidopsis* seedlings with GA<sub>3</sub> had no effect on the genes involved in the flavonoid/anthocyanin synthesis studied (Fig. 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* (Fig. 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). In addition to having anthocyanin as endproduct, 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 sixfold *AAT* induction was triggered by GA<sub>3</sub> when compared with the control (Fig. 3). These two genes are not involved in the main pathway of anthocyanin synthesis.

### Synergistic effect of JA and sucrose on anthocyanin synthesis genes

To gain additional clues to the effect of JA on anthocyanin synthesis, 4-d old *Arabidopsis* seedlings were treated with JA or left untreated. Although the positive effect of JA on anthocyanin accumulation is well known (Table 1), we found that JA alone did not affect the biosynthetic pathway in *Arabidopsis* seedlings (Fig. 3). The expression of the transcription factors *PAP1* and *PAP2* were not enhanced by JA either (Fig. 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 JA 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 (Fig. 3).

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

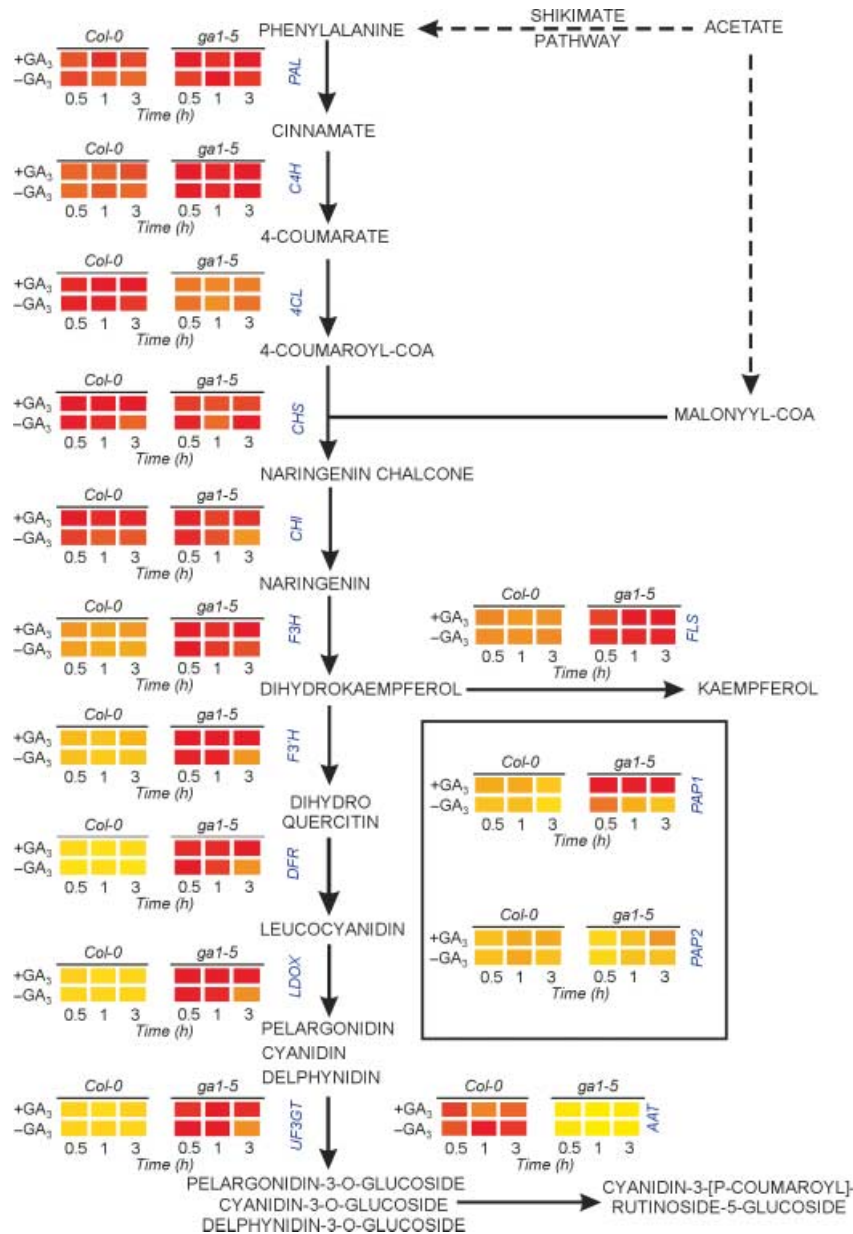
Since feeding *Arabidopsis* seedlings concomitantly with sucrose and ABA resulted in an increased level of *DFR* expression and in a higher level of anthocyanin(s) (Fig. 1a), the role of ABA was investigated in more detail. The mRNA accumulation of transcripts related to flavonoid/anthocyanin synthesis in ABA-enriched media, with or without sucrose, was analysed. The results indicated that ABA alone did not have effect on any of the genes analysed except for *AAT* and *PAP2* (Fig. 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, an enhancement in the sucrose-induction of most of the genes involved in the anthocyanin biosynthesis was observed (Fig. 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 (Fig. 3).

### Gibberellin 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 catalysing conversion of geranylgeranyl pyrophosphate (GGPP) to copalyl pyrophosphate (CPP). This GA-deficient mutant germinates without GA treatment (Koornneef & van der Veen, 1980), allowing analysis of the consequences of a low endogenous GA 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 analysed and the results are reported in Fig. 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 (3 h) 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 GA concentration 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 GA-dependent repression of *dihydroflavonol reductase*

To gain further insight into the possible interaction between sucrose and GA, we investigated the effect of sucrose in the



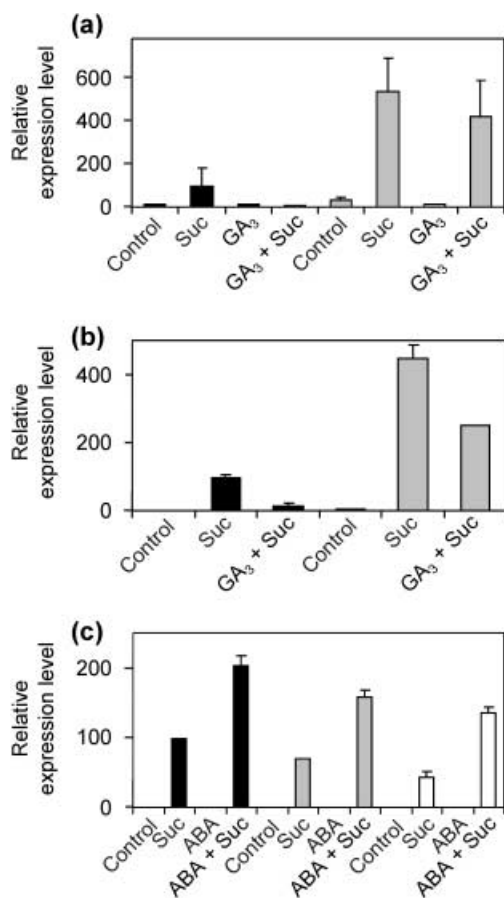
**Fig. 4** Effect of gibberellin (GA) on the *ga1-5* mutant. The AtGenExpress dataset TAIR-ME00343 (GA<sub>3</sub> time-course in *Col-0* and *ga1-5* mutant seedlings) was used. The dataset was obtained using 7-d-old *Arabidopsis* seedlings grown for 0.5, 1, 3 h on Murashige and Skoog medium (–GA<sub>3</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) indicates 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. Each set of expression data referring to each gene should be viewed individually, since scaling of colour shades is done based on the higher (red) and lower (yellow) expression data relative to the gene under study.

presence/absence of GA<sub>3</sub> on the GA-insensitive mutant *gai*, a *GAI* gain-of-function mutant impaired in GA(s) signalling (Wilson & Somerville, 1995). First, the effect of sucrose supplied with or without GA<sub>3</sub> was tested 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 (Fig. 5a), as observed in the *Columbia-0* *Arabidopsis* ecotype (Fig. 2a). Conversely, feeding the *gai* mutant seedlings with sucrose and GA<sub>3</sub> resulted in an expression of *DFR* comparable to that of sucrose alone (Fig. 5a). This would seem to prove that *GAI* signalling is involved in the crosstalk between sucrose and GA in wild-type *Arabidopsis* seedlings. This was replicated and confirmed using leaves from

*Arabidopsis* adult plants (Fig. 5b). The induction triggered by sucrose in seedlings (Fig. 5a) and leaf discs (Fig. 5b) was much stronger in *gai*, which was less sensitive to the addition of GA<sub>3</sub> (Fig. 5a,b).

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

To better understand the role of ABA in anthocyanin accumulation the responses of the ABA-deficient mutant (*aba1-3*), impaired in the epoxidation of zeaxanthin and antheraxanthin to violaxanthin (Rock & Zeevaart, 1991), together with the ABA-insensitive (*abi1-1*; Koornneef *et al.*, 1984) mutant to ABA, with and without sucrose were analysed. The expression



**Fig. 5** Effect of sucrose (Suc), gibberellic acid ( $GA_3$ ) and abscisic acid (ABA) in *Arabidopsis* mutants. (a) *DFR* mRNA level in seedlings (4 d old) of the wild-type (*Ler*; closed bars) and *gai* (tinted bars) mutant. (b) *DFR* mRNA level in leaf discs of the wild-type (*Ler*; closed bars) and *gai* (tinted bars) mutant. (c) *DFR* mRNA level in seedlings (4 d old) of the wild-type (*Ler*; closed bars) and *aba1-3* (tinted bars) and *abi1-1* (open bars) mutants. Experimental conditions: (a,c) 4-d-old *Arabidopsis* seedlings were grown for 24 h on a sucrose-free Murashige and Skoog (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 24 h on a sucrose-free MS medium (Control) or MS medium supplemented with sucrose or  $GA_3$  with sucrose. Relative expression level (REL), measured by real-time reverse transcription polymerase chain reaction (RT-PCR), is shown. (REL: 100 = expression data from the sucrose-treated *Ler* sample). The results are mean  $\pm$  SD ( $n = 2$ ).

levels of *DFR* in *aba1-3*, *abi1-1*, and in the wild-type *Landsberg erecta* was analysed after sucrose, and after sucrose with ABA treatments (Fig. 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, the effect of this sugar in the presence/absence of ABA on the abscisic acid-insensitive mutant (*abi1-1*) was investigated. The results showed that the synergistic effect of ABA and sucrose was retained in *abi1-1*, indicating the ABI1 is not required for the effects of ABA on the sucrose-induction anthocyanin pathway (Fig. 5c).

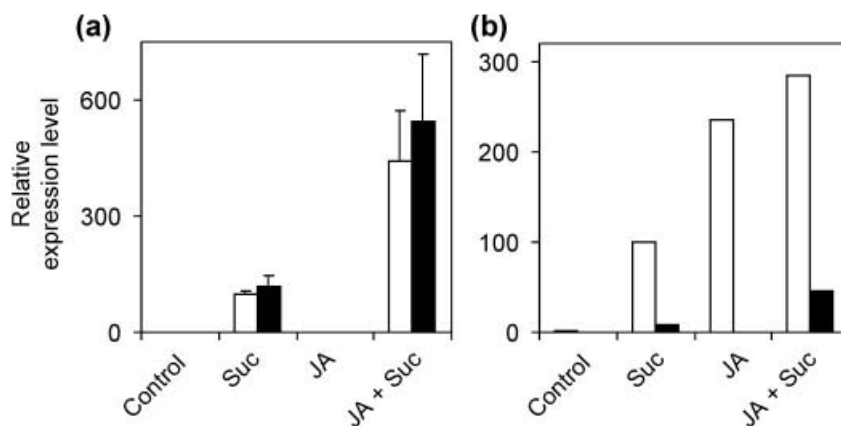
#### *COI1* is involved in the sucrose-dependent induction of dihydroflavonol reductase

Mutants defective in JA-signalling include *coronatine insensitive 1 (coi1)* and *jasmonate-insensitive 1 (jar1)* mutant (Staswick *et al.*, 1992, 2002; Feys *et al.*, 1994). 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 & 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. The results showed that the induction triggered by sucrose is retained in *jar1-1* (Fig. 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 JA-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 JA, a likely consequence of the higher sugar content of photosynthetic tissues when compared with germinating seedlings. The effect of JA 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 (Fig. 6b).

#### 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), although some discrepancies might be easily explained taking into account the different plant species and tissues analysed (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



**Fig. 6** Role of *JAR1* and *COI1* in the sucrose-induction of *DFR*. (a) *DFR* mRNA level in 4-d-old *Col-0* (open bars) and in the *jar1-1* (closed bars) *Arabidopsis* seedlings grown for 24 h on a sucrose-free MS medium (Control) or MS medium supplemented with sucrose (Suc) and/or jasmonate (JA). (b) *DFR* mRNA level in *Col-gl* (open bars) and *coi1-1* (closed bars) plants. Leaf strips from *Col-gl* and *coi1-1* plants were used in this experiment. See the Material and Methods section for the procedure used to identify homozygous *coi1-1* mutants. Relative expression level (REL), measured by real-time reverse transcription polymerase chain reaction (RT-PCR), is shown (REL: 100 = expression data from the sucrose-treated *Col* samples).

*DFR* and genes downstream of it (Solfanelli *et al.*, 2006). It is presently unknown whether the effects of sucrose result from a direct sucrose-sensing mechanism(s) or from the consequences of sucrose metabolism. 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 hexoses 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 (Fig. 1a,b). Our results indicated that GAs, JA and ABA, but not 2,4D, ethylene and cytokinins, may interact or crosstalk with sucrose to form a complex web of overlapping signalling pathways that coordinate anthocyanin accumulation.

Gibberellins counteracted the sucrose induction of anthocyanin biosynthesis (Figs 1a,b, 2a, 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 GA<sub>3</sub> (Fig. 3). Genes acting downstream of naringenin are upregulated in the *gai-5* mutant, indicating that low endogenous GA concentrations favour the expression of the anthocyanin pathway genes (Fig. 4). Interestingly, the expression of *AAT* is, instead, lower in the *gai-5* mutant (Fig. 4), in agreement with the positive effects of GA<sub>3</sub> on the expression of this gene (Fig. 3, Table S2). The use of *gai* mutant seedlings (Peng *et al.*, 1997) revealed that *GAI* is involved in the crosstalk between sucrose and GAs (Fig. 5a,b). Jiang *et al.* (2007) detected low-level *F3H*, *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 (Fig. 5a,b), suggesting that GAs 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 JA family of signalling molecules regulates responses to many biotic and abiotic stresses (Turner *et al.*, 2002; Devoto & Turner, 2003; Balbi & Devoto, 2007). Moreover, JAs may also stimulate anthocyanin accumulation in many plant systems (Table 1). Jasmonic acid enhanced the sucrose-induction of the entire biosynthetic pathway, including the two transcription factors *PAP1* and *PAP2* (Fig. 3, Table S2). The synergistic effect was present at mRNA level (Figs 2b, 3) and correlates with anthocyanin accumulation (Fig. 1a). In agreement with our results, feeding corn (*Zea mays*) leaves with JA and sucrose resulted in enhanced anthocyanin content (Kim *et al.*, 2006). The ability of JA to induce several anthocyanin genes in *Arabidopsis* 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 MS 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 prerequisite for the action of JA. It was recently shown that the JA-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 (Fig. 6), suggesting a convergence of the sucrose and JA signalling pathways.

In several plant systems ABA induced anthocyanin accumulation, whereas in others it had a repressive role (Table 1). Abscisic acid cosupplied 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 *CI* 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 *CI* 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 (Fig. 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 (Fig. 5c). The use of other ABA biosynthetic (*aba2/aba3*) and insensitive (*abi4/abi5*) mutants would help in expanding our knowledge on the interaction between sucrose and ABA.

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 (Skp, Cullin, F-box) ubiquitin ligase, is involved in the response to JA (Santner & Estelle, 2007; Thines *et al.*, 2007) and interacts with the *Arabidopsis* KIN10 and KIN11, two SNF1-related protein kinases (SnRK) (Farras *et al.*, 2001). Interestingly, sucrose represses KIN10, a SnRK1 that, when overexpressed, 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 JA 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 & Estelle, 2007), triggering derepression 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 GA 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 (Fig. 5a,b). Gibberellin-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 prerequisite 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. It has been demonstrated that ABA increases the stability of RGA and blocks its GA-induced degradation (reviewed by Weiss & Ori, 2007). Therefore, ABA may act as a repressor of the GA-pathway and derepress the synthesis of anthocyanin genes. Further research is thus required to elucidate these interacting regulative networks.

Overall, our results indicated that a crosstalk between sucrose and hormones controls anthocyanin biosynthesis in *A. 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 prerequisite for the action of plant hormones to regulate anthocyanin accumulation.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Table S1** List of primers and Taqman probes used in real time polymerase chain reaction (PCR) analysis

**Table S2** Effect of sucrose and gibberellic acid ( $GA_3$ ), jasmonic acid (JA), and abscisic acid (ABA) on the expression of genes involved in anthocyanin biosynthesis. Four-day old

*Arabidopsis* seedlings were grown for 24 h on a sucrose-free MS medium (control) or standard medium supplemented with sucrose/hormones alone or fed together. Treatments were as follows: control, sucrose, hormone (GA or ABA or JA), hormone + sucrose. Relative expression level (REL), measured by real-time reverse transcription polymerase chain reaction (RT-PCR), is shown. Data are mean  $\pm$  SD ( $n = 2$ ). (REL: 100 = expression data from the sucrose-treated sample)

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