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ORIGINAL ARTICLE

Regulation of anthocyanin biosynthesis in *Arabidopsis thaliana* red *pap1-D* cells metabolically programmed by auxins

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Abstract Red *pap1-D* cells of *Arabidopsis thaliana* have been cloned from *production of anthocyanin pigmentation 1-Dominant* (*pap1-D*) plants. The red cells are metabolically programmed to produce high levels of anthocyanins by a WD40-bHLH-MYB complex that is composed of the TTG1, TT8/GL3 and PAP1 transcription factors. Here, we report that indole 3-acetic acid (IAA), naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) regulate anthocyanin biosynthesis in these red cells. Seven concentrations (0, 0.2, 0.4, 2.2, 9, 18 and 27 μM) were tested for the three auxins. IAA and 2,4-D at 2.2–27 μM reduced anthocyanin levels. NAA at 0–0.2 μM or above 9 μM also decreased anthocyanin levels, but from 0.4 to 9 μM, it increased them. HPLC–ESI–MS analysis identified seven cyanin molecules that were produced in red *pap1-D* cells, and their levels were affected by auxins. The expression levels of ten genes, including six transcription factors (*TTG1*, *EGL3*, *MYBL2*, *TT8*, *GL3* and *PAP1*) and four pathway genes (*PAL1*, *CHS*, *DFR* and *ANS*) involved in anthocyanin biosynthesis were analyzed upon various auxin treatments. The resulting data showed that 2,4-D, NAA and IAA control anthocyanin biosynthesis by regulating the expression of *TT8*, *GL3* and *PAP1* as well as genes in the anthocyanin biosynthetic pathway, such as *DFR* and *ANS*. In addition, the expression of *MYBL2*, *PAL1* and *CHS* in red *pap1-D* and wild-type cells differentially respond to the three auxins. Our data demonstrate that the three auxins regulate anthocyanin biosynthesis in metabolically programmed red cells via altering the expression of transcription factor genes and pathway genes.

Keywords Anthocyanin biosynthesis · *Arabidopsis thaliana* · Auxins · Metabolic engineering · *pap1-D* cells · WBM complex · Regulation route

Introduction

Anthocyanins are a large group of natural pink, red, purple or blue flavonoid pigments that are widely produced in crops and fruit plants (Harborne and Baxter 1999). In addition to providing natural color and protecting plants from UV radiation, anthocyanins are important nutraceuticals. Numerous investigations have demonstrated that anthocyanins have antioxidative (Kano et al. 2005; Pool-Zobel et al. 1999; Denev et al. 2010; Shimizu et al. 2010; Tsuda et al. 1994), anti-cancer (Bowen-Forbes et al. 2010; Barrios et al. 2010; Dai et al. 2009; Faria et al. 2010) and neuron-protective properties (Bhuiyan et al. 2012). They are present in fruits such as blueberries (Barnes et al. 2009; Koca and Karadeniz 2009), grapes (Wang et al. 2003; Jeong et al. 2004) and strawberries (Silva et al. 2002; Given et al. 1988), and provide high nutritional value to these fruits and their corresponding products. Accordingly, numerous efforts have been carried out to understand anthocyanin structures and biosynthesis (Holton and Cornish 1995;

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Butelli et al. 2008; Clifford 2000) to improve the nutritional value of various crops.

We have previously engineered red *pap1-D* cells from the leaves of *production of anthocyanin pigmentation 1-Dominant* (*pap1-D*) plants (Shi and Xie 2011), which were generated by T-DNA activation tagging (Borevitz et al. 2000). This dominant mutant plant line contains four cauliflower mosaic virus 35S enhancer sequences that have been inserted into the region immediately adjacent to *PAP1*. This insertion results in high expression of *PAP1*, which encodes the MYB75 transcription factor, thus leading to high anthocyanin accumulation in leaves and other tissues. In the past decade, numerous investigations using this plant line have demonstrated that on the one hand, PAP1 is a master regulator of anthocyanin biosynthesis (Xie et al. 2006; Solfanelli et al. 2006; Zhou et al. 2008, 2012 Gonzalez et al. 2008; Zvi et al. 2012; Shi and Xie 2011); on the other hand, anthocyanin biosynthesis regulated by PAP1 is impacted by different abiotic factors (such as temperature, light, nitrogen and nitrate) (Shi and Xie 2010; Rowan et al. 2009). In addition, the expression of other transcription factors, such as bHLH genes (*TT8*, *transparent testa 8*; *TT2*, *transparent testa 2*; *GL3*, *glabra 3*; and *EGL3*, *enhancer glabra 3*) and WD40 genes (*TTG1*, *transparent testa glabra 1*) (Ramsay and Glover 2005; Zhang et al. 2003; Gonzalez et al. 2008; Shi and Xie 2010, 2011; Xie and Shi 2012), affects anthocyanin biosynthesis regulated by PAP1. We have utilized this plant to clone red *pap1-D* cells, which have allowed us to characterize genes encoding the regulatory complexes involved in anthocyanin biosynthesis. Our previous genome-wide microarray analysis, together with quantitative and semi-quantitative RT-PCR analyses, identified transcription factors and pathway genes of the anthocyanin biosynthesis in red *pap1-D* cells (Shi and Xie 2011). These results are summarized in Fig. 1. The data demonstrated that *PAP1*, but not its homologs (*PAP2, production of anthocyanin pigmentation 2*; *TT2; MYB113 and MYB114*), is expressed in red *pap1-D* cells. In three bHLH members, *TT8* is expressed in red *pap1-D* cells, but not in wild-type control cells, which lack anthocyanins. The expression level of *GL3* is higher in red *pap1-D* cells than in wild-type cells. These experiments have indicated that anthocyanin biosynthesis in red *pap1-D* cells is controlled by the TTG1 (WD40)-TT8/GL3 (bHLH)-PAP1 (MYB75) complex (Fig. 1), but not by other complexes (Shi and Xie 2011; Zhou et al. 2012; Xie and Shi 2012). The transcription of *DFR*, *ANS* and two *GT* genes is activated in red *pap1-D* cells, which is consistent with the upregulation of this complex. In addition, the expression levels of early pathway genes including *CHS*, *CHI*, *F3H* and *F3′H* are significantly increased in red *pap1-D* cells compared to wild-type cells. Furthermore, red *pap1-D* cells express a large number of genes, the expression levels

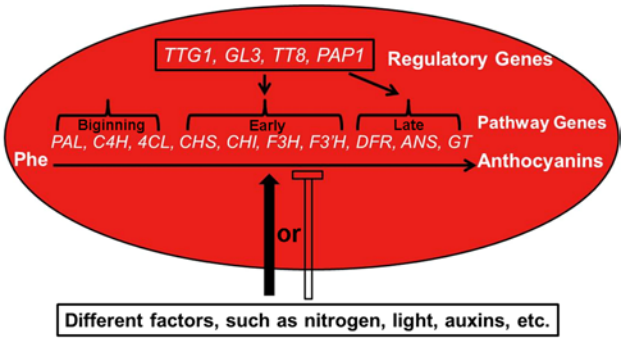

Fig. 1 The biosynthetic pathway and regulatory genes of anthocyanin biosynthesis in red *pap1-D* cells. The pathway genes include the beginning steps genes, *PAL* phenylalanine ammonia lyase, *C4H* cinnamate-4-hydroxylase, *4CL* 4-coumaryol CoA ligase; the early genes, *CHS* chalcone synthase, *CHI* chalcone isomerase, *F3H* flavanone-3 hydrolase, *F3′H* flavonoid-3′-hydroxylase; and the late genes, *DFR* dihydroflavonol reductase, *ANS* anthocyanidin synthase (also called *LDOX* leucoanthocyanidin dioxygenase) and *GT* glycotransferase. The regulatory genes include *GL3* glabra 3, *PAP1* production of anthocyanin pigmentation 1, *TT8* transparent testa 8, *TTG1* transparent glabra 1. The *solid blank bar arrow* and the empty "T" shape indicate that various factors can up-regulate or down-regulate the biosynthetic pathway genes

of which are either undetectable or very low in control cells (Shi and Xie 2011).

Red *pap1-D* cells are an appropriate system for understanding anthocyanin biosynthesis that is regulated by various factors. As has been reported in the literature for many species, environmental conditions such as temperature, light and nutrients regulate anthocyanin biosynthesis (Gortner 1918; Harrow 1930; Blank 1947; Timberlake and Bridle 1975; Wellmann et al. 1976; Harborne and Grayer 1988; Holton and Cornish 1995). However, the mechanisms behind this natural phenomenon remain largely elusive. For example, *A. thaliana* has been demonstrated to contain various WBM complexes that control anthocyanin biosynthesis (Gonzalez et al. 2008; Shi and Xie 2011). However, questions regarding how these complexes respond to environmental factors and whether they depend upon each other in a single cell remain unanswered. In addition, whether different regulatory complexes respond to environmental conditions in the same manner is unknown. To address these questions, a large number of investigations have been carried out in both callus and suspension cell cultures under controlled conditions in the past several decades. Carrot callus and suspension cell cultures have been used as model systems (Dougall et al. 1980, 1983; Ozeki and Komamine 1985, 1986; Yoshihiro and Atsushi 1985; Narayan et al. 2005). Other cell suspension systems investigated include those for grapes (Do and Cormier 1991a, b; Bao Do and Cormier 1991; Cormier et al. 1992) and strawberries (Mori et al. 1993, 1994; Mori and Sakurai 1994).

Investigations using these systems have shown that various factors, such as auxins, cytokinins, light/dark, temperature, nutrients and sucrose, can increase or decrease anthocyanin production. Efforts are still underway to use these model systems and other plant cell cultures. Unfortunately, little progress has been made in elucidating the mechanisms involved in the regulation of anthocyanin biosynthesis in these systems. For example, regulatory and biosynthetic genes remain uncharacterized. Recently, we have shown that these limitations can be overcome using the red *pap1-D* cell system. Unlike other research systems, anthocyanin biosynthesis in red *pap1-D* cells (calli) is controlled by the expression of *TTG1*, *TT8*, *GL3* and *PAP1*, whose products form a regulatory complex as described above. The mechanisms by which various factors regulate anthocyanin biosynthesis can be determined via analyses of these genes. For example, red *pap1-D* cells have allowed us to characterize the regulation of anthocyanin biosynthesis by nitrogen. We demonstrated that high concentrations of total nitrogen, particularly the ammonia form, strongly decrease anthocyanin production. Gene expression analysis revealed that high nitrogen concentrations and ammonia strongly down-regulated or inactivated the expression of *PAP1* and *TT8*, most likely leading to reduced levels of the TTG1-TT8/GL3-PAP1 complex. Accordingly, the expression levels of *PAL1*, *CHS*, *DFR* and *ANS* (Fig. 1) were significantly decreased, leading to a reduction in anthocyanin levels. Our experiments demonstrated that various nitrogen forms and concentrations control anthocyanin biosynthesis likely in three steps: the alteration of TTG1-TT8/GL3-PAP1 complex levels, the alteration of the expression of key genes in the biosynthetic pathway, and then alterations in anthocyanin production (Zhou et al. 2012). In addition, we have observed that other factors, such as auxins and light, affect anthocyanin production (Fig. 1).

Auxin is a main phytohormone involved in almost every aspect of plant development, such as embryogenesis, root initiation, leaf development, stem elongation and plant stress responses, as reviewed in a few recent articles (Yoshida et al. 2013; Strader and Nemhauser 2013; Rahman 2013). Although the effects of auxins on secondary metabolism in planta are not well understood, numerous investigations have determined that auxins control anthocyanin biosynthesis in cultured plant cells in vitro. For example, previous investigations on anthocyanin biosynthesis in suspension-cultured carrot cells (Ozeki and Komamine 1985, 1986; Narayan et al. 2005), *Oxalis linearis* (Meyer and Vanstaden 1995) and strawberry (Mori et al. 1994) have shown that certain levels of 2,4-dichlorophenoxyacetic acid (2,4-D) strongly inhibit anthocyanin formation. In addition, we observed inhibitory effects of 2,4-D on anthocyanin formation in red *pap1-D* cells (Shi and Xie 2011). To date, the mechanisms of anthocyanin biosynthesis regulated by 2,4-D and other auxins remain elusive. In the present report, we characterize the regulation of anthocyanin biosynthesis in *pap1-D* cells treated with various concentrations of 2,4-D, naphthaleneacetic acid (NAA) and indole 3-acetic acid (IAA). Seven different concentrations of the three auxins were tested. HPLC–ESI–MS was used to characterize the anthocyanin profiles upon each treatment. The expression profiles of ten representative genes were analyzed by semi-quantitative RT-PCR. The resulting data showed that the regulation of anthocyanin biosynthesis by auxins occurred via alterations in the expression of *PAP1*, *TT8* and other genes. The expression of genes encoding proteins for the formation of the TTG1-TT8/GL3-PAP1 complex is regulated by auxins.

Materials and methods

Reagents

Kinetin, 2,4-D, NAA, IAA, sucrose, phytoagar, macronutrients, micronutrients and organic nutrients used in the MS medium were purchased from Plant Media (Dublin, OH, USA). The cyanidin standard was purchased from Indofine (Hillsborough, NJ, USA). Hydrochloric acid (36.5–38 %) was purchased from BDH (cat# BHH3028-2.5L, Westchester, PA, USA). Acetonitrile (LC–MS grade) was purchased from EMD (cat# AX0156-1, Gibbstown, NJ 08027, USA). Acetic acid (HPLC grade, cat# 9515-03) and methanol (LC–MS grade, cat# 9830-03) were purchased from J. T. Baker (Phillipsburg, NJ 08865, USA).

Medium preparation and callus culture

The medium preparation and callus maintenance were as described previously (Shi and Xie 2011). Briefly, red *pap1-D* and wild-type calli were maintained on an agar-solidified medium composed of a modified MS medium (Murashige and Skoog 1962) supplemented with 3 % sucrose, 0.47 μM (0.1 mg/l) 2,4-D and 1.13 μM (0.25 mg/l) kinetin. In our modified MS medium, ammonium nitrate (NH_4NO_3) was removed, and the concentration of potassium nitrate (KNO_3) was reduced to 9.4 mM.

To test the effects of 2,4-D, IAA and NAA on anthocyanin biosynthesis, the 0.47 μM 2,4-D used in the maintenance medium was replaced with seven concentrations (0, 0.2, 0.4, 2.2, 9, 18 and 27 μM) of each auxin. The seven concentrations were selected mainly based on our medium optimization experiments reported previously (Shi and Xie 2011). The absence (indicated by 0 μM) of auxin in medium was used as control. The kinetin concentration was set at 1.13 μM. All media were solidified with 0.8 % phytoagar, adjusted to pH 5.8 and autoclaved for 20 min. Twenty

Table 1 Primer pairs and gradient thermal programs designed for RT-PCR to amplify cDNA fragment of ten genes involved in anthocyanin biosynthesis and *ACTIN2* as control reference

Gene name	Primer sequence (5′–3′)	PCR program
PAL1	Forward: 5′-AGTGGACGCTATGTTATGC-3′	94 °C × 3′ (94 °C × 30″, 51 °C × 30″, 72 °C × 1′20″) × 25, 72 °C 10′
	Reverse: 5′-GATTATCGTTGACGGAGTTA-3′	
CHS	Forward: 5′-CAAGCGCATGTGCGACAA-3′	94 °C × 3′ (94 °C × 30″, 51 °C × 30″, 72 °C × 1′) × 25, 72 °C 10′
	Reverse: 5′-TCCCTCAAATGTCCGTCT-3′	
DFR	Forward: 5′-TTTCCCAAAGCACAATCT-3′	94 °C × 3′ (94 °C × 30″, 51 °C × 30″, 72 °C × 40″) × 25, 72 °C 10′
	Reverse: 5′-ACACGAAATACATCCATCC-3′	
ANS	Forward: 5′-ACGGTCCTCAAGTTCCCACA-3′	94 °C × 3′ (94 °C × 25″, 57 °C × 25″, 72 °C × 30″) × 25, 72 °C 10′
	Reverse: 5′-TCGCGTACTCACTCGTTGCTTCTAT-3′	
ACTIN2	Forward: 5′-GTTGGGATGAACCAGAAGGA-3′	94 °C × 3′ (94 °C × 15″, 50 °C × 15″, 72 °C × 15″) × 25, 72 °C 10′
	Reverse: 5′-GAGGAGCCTCGGTAAGAAGA-3′	
TTG1	Forward: 5′-TCGTCATGTGCGTAAGTAGGTT-3′	94 °C × 3′ (94 °C × 15″, 51 °C × 15″, 72 °C × 20″) × 25, 72 °C 10′
	Reverse: 5′-GTCCAATAAAGCAAGACAGGGT-3′	
PAP1	Forward: 5′-TGAAAAAGAGAGACATTACGCCCAT-3′	94 °C × 3′ (94 °C × 15″, 51 °C × 15″, 72 °C × 25″) × 25, 72 °C 10′
	Reverse: 5′-TCGCTTCAGGAACCAAAATATCTACC-3′	
TT8	Forward: 5′-CCGTCGTCACAATGGGTGC-3′	94 °C × 3′ (94 °C × 15″, 58 °C × 15″, 72 °C × 15″) × 28, 72 °C 10′
	Reverse: 5′-CCTCCTGCGTCGCTCTGCTA-3′	
GL3	Forward: 5′-TCGGTTCGTTTGGTAATGAGG-3′	94 °C × 3′ (94 °C × 15″, 50 °C × 15″, 72 °C × 15″) × 28, 72 °C 10′
	Reverse: 5′-GCTTGCAATTGACGGTTAAGC-3′	
EGL3	Forward: 5′-CATCAGCTAATACTCGGACCGC-3′	94 °C × 3′ (94 °C × 15″, 50 °C × 15″, 72 °C × 15″) × 28, 72 °C 10′
	Reverse: 5′-TCTGCGATTTCTCTCCCAATGT-3′	
MYBL2	Forward: 5′-ATGAACAAAACCCGCCTTCG-3′	94 °C × 3′ (94 °C × 15″, 51 °C × 15″, 72 °C × 40″) × 25, 72 °C 10′
	Reverse: 5′-TCATCGGAATAGAAGAAGCG-3′	

milliliters of warm, unsolidified agar medium was poured into each petri dish (15 × 100 mm, height × diameter) and allowed to solidify at room temperature.

Ten petri dishes were prepared for every concentration treatment of each auxin. Five of these were inoculated with red *pap1-D* calli, and the remaining five were inoculated with wild-type calli as controls. Each plate was inoculated with 0.3 g fresh weight of calli. The petri dishes were then placed in a tissue culture chamber. The photoperiod, light intensity and temperature were 16/8 h (light/dark), 50 μmol/m/s and 22 °C, respectively. After 15 days of culture, calli from each plate were harvested, and the fresh weights were measured. The calli were then frozen in liquid nitrogen and stored at −80 °C. The experiment was repeated twice.

Reverse transcription-polymerase chain reaction

The frozen calli were homogenized into fine powder in mortars in liquid nitrogen. DNA-free total RNA was isolated from 0.1 g of frozen calli powder using the Qiagen RNeasy Plant Mini Kit and on-column RNase-free DNase I (Qiagen, Germantown, MD, USA) following the manufacturer's protocols. Two micrograms of total RNA was used for cDNA synthesis. Reverse transcription was performed using the SuperScript® III first-strand synthesis system (Invitrogen, Grand Island, NY, USA). Gene-specific primers were designed to amplify the cDNA fragments of the target genes. Polymerase chain reactions were carried out on a thermometer (Mastercycler® ep, Eppendorf) to amplify the cDNA fragments of ten genes involved in anthocyanin biosynthesis using *ACTIN2* as a reference. The primers and thermal gradient programs for each gene analyzed are listed in Table 1. The PCR products were examined by electrophoresis on 1 % agarose gels and imaged using a Bio-Rad image system.

Anthocyanin extraction and measurement

A total of 100 mg of frozen calli powder for each sample was suspended in 1 ml of extraction buffer (0.5 % HCl in 100 % methanol) contained in a 1.5 ml extraction tube. The methods for the extraction and quantification of anthocyanins using a wavelength of 530 nm were as described previously (Shi and Xie 2011).

HPLC–PDA-ESI–MS analysis

Anthocyanins extracted in 0.1 % HCl in 100 % methanol were profiled using HPLC–PDA-ESI–MS on a 2010EV

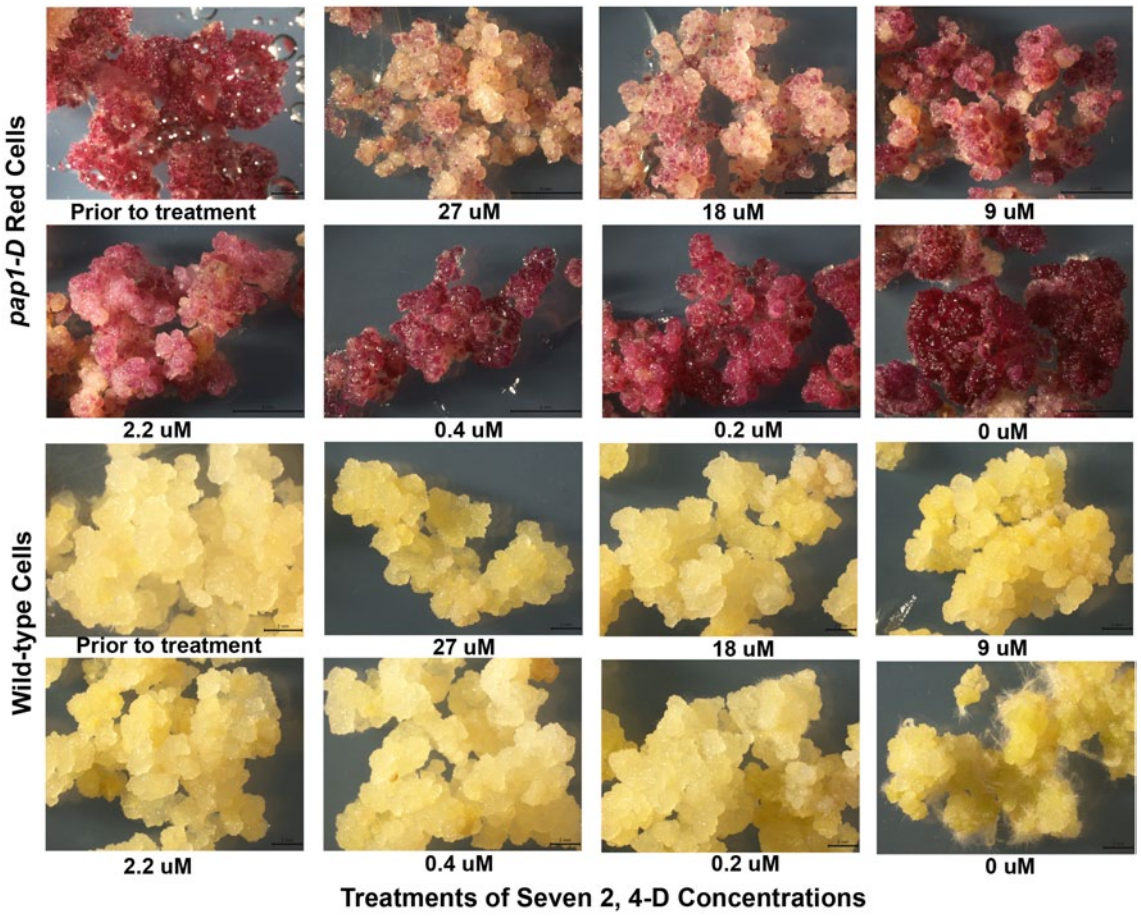

Fig. 2 The effects of seven different concentrations of 2,4-D on the red pigmentation of calli

LC/UV/ESI/MS instrument (Shimadzu, Japan). All of the methods and parameters used for the analysis were as described previously (Shi and Xie 2011).

Statistical analysis

Student's *T* test (*P* value <0.05) was used to evaluate the significance of anthocyanin levels upon the auxin treatments.

Results

Effects of auxins on anthocyanin pigmentation and production in red *pap1-D* calli

The three auxins tested resulted in altered pigmentation of red *pap1-D* cells (Figs. 2, 3, 4). Of the seven concentrations tested, 2.2, 9, 18 and 27 μM 2,4-D strongly reduced the red pigmentation of the calli (Fig. 2). The biomasses of both red *pap1-D* and wild-type calli were slightly lower when treated with high concentrations of 2,4-D compared to lower concentrations (S-Fig. 1). The quantification data showed that the total anthocyanin production in red *pap1-D* calli was significantly reduced upon 2,4-D treatment in the concentration range of 2.2–27 μM (Fig. 5a).

NAA also altered anthocyanin pigmentation and production in red *pap1-D* calli (Figs. 3, 5b). From 0 to 27 μM, the intensity pattern of anthocyanin pigmentation decreased from 0 to 0.2 μM, increased from 0.2 to 9 μM and then decreased from 9 to 27 μM (Fig. 3). The biomasses of both red *pap1-D* and wild-type calli at 18 and 27 μM NAA were slightly lower than at other concentrations (S-Fig. 1). The anthocyanin quantification data were consistent with the red pigmentation patterns of the calli (Fig. 5b). A peak anthocyanin level was obtained at 9 μM NAA.

IAA moderately reduced anthocyanin biosynthesis in red *pap1-D* calli (Figs. 4, 5c). Compared to 0 μM, the six other concentrations of IAA led to reductions in the pigmentation of the calli (Fig. 4). The biomasses of both red *pap1-D* and wild-type calli at 18 and 27 μM IAA were slightly lower than at the other concentrations (S-Fig. 1). The anthocyanin quantification data at 530 nm (Fig. 5c) were consistent with pigmentation patterns.

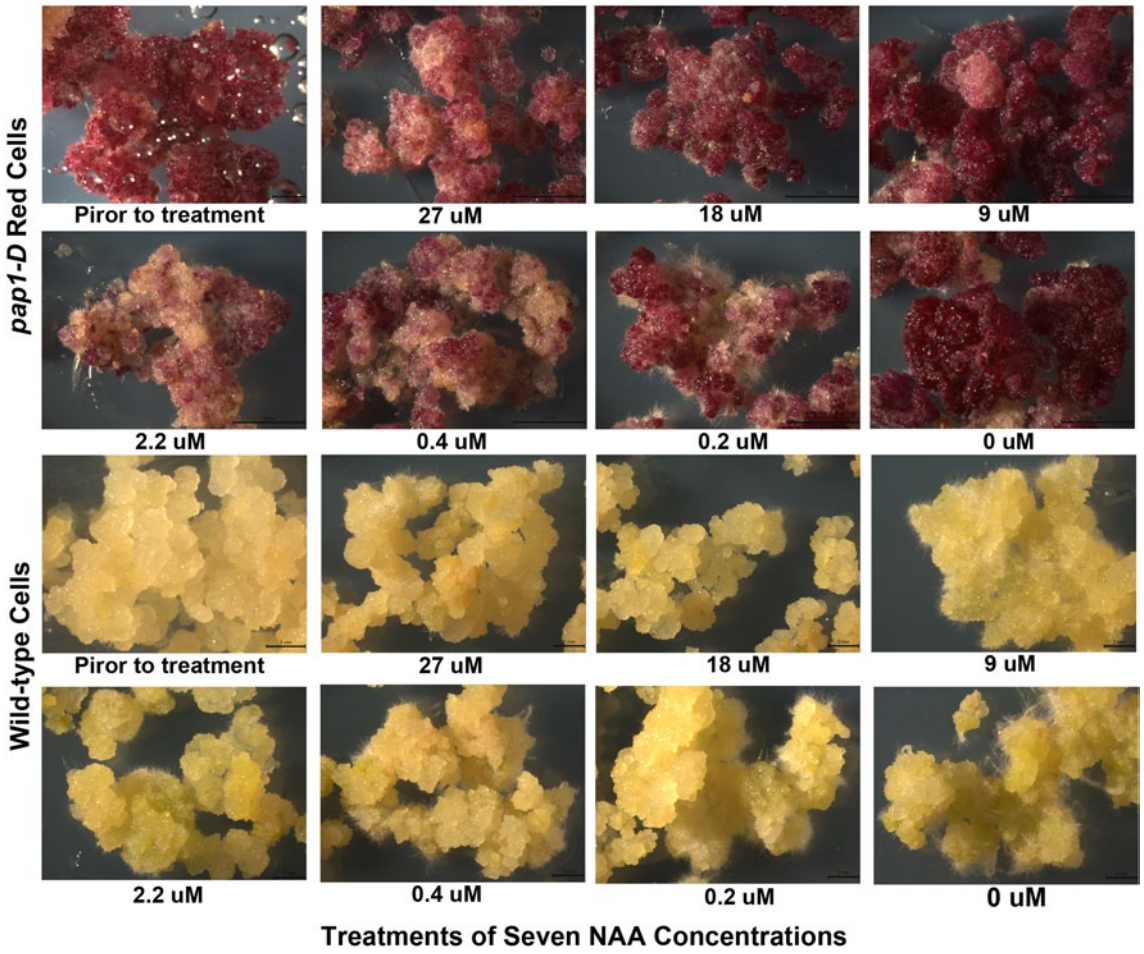

Fig. 3 The effects of seven different concentrations of NAA on the red pigmentation of calli

In contrast, the wild-type cells did not produce anthocyanins at any of the seven concentrations of 2,4-D (Figs. 2, 5a), NAA (Figs. 3, 5b) and IAA (Figs. 4, 5c).

HPLC–PDA-ESI–MS profiling of anthocyanins

The anthocyanin profiles in red *pap1-D* cells were characterized using HPLC–PDA-ESI–MS analysis. As we reported previously (Shi and Xie 2011; Zhou et al. 2012), a positive mode was used to ionize anthocyanins and characterize their mass spectrum properties. In all of the experiments using various concentrations of NAA, IAA or 2,4-D, the profiles of the anthocyanin peaks in the red *pap1-D* cells were the same as reported previously (Shi and Xie 2011). Seven peaks with one main peak (peak 6) were detected at 530 nm by a photodiode array detector (Fig. 6). Peaks 1, 3, 4, 5, 6 and 7 were assigned to the *Arabidopsis* anthocyanin molecules A2 (cyanidin 3-*O*-[2″-*O*-(xylosyl) glucoside] 5-*O*-(6‴-*O*-malonyl) glucoside), *cis*-A3, A3 (cyanidin 3-*O*-[2″-*O*-(xylosyl) 6″-*O*-(*p*-coumaroyl) glucoside] 5-*O*-glucoside), *cis*-A5, A5 (cyanidin 3-*O*-[2″-*O*-(xylosyl)-6″-*O*-(*p*-coumaroyl) 5-*O*-malonyl-glucoside), and A16 (methylated form of A5), respectively (Shi and Xie 2011; Zhou et al. 2012). The compound of peak 2 is likely the methylated form of A2. In contrast, no anthocyanins were detected in the wild-type cells under any treatments (S-Fig. 2).

Alterations of anthocyanin profiles at different concentrations of the three auxins

Chromatographs of the anthocyanins in red *pap1-D* cells under all of the treatments were recorded at 530 nm to show responses of profile to different concentrations of auxins. The values of both the areas and heights of the anthocyanin peaks were used to compare the effects of the seven different concentrations of the three auxins on profiles.

Compared to 0 μM (control), all other six concentrations of 2,4-D decreased the area and height values of the seven anthocyanin peaks in red *pap1-D* cells (Fig. 6). In particular, 2,4-D at 27 μM led to the smallest area and height values of all the peaks (Fig. 6). All peaks were simultaneously

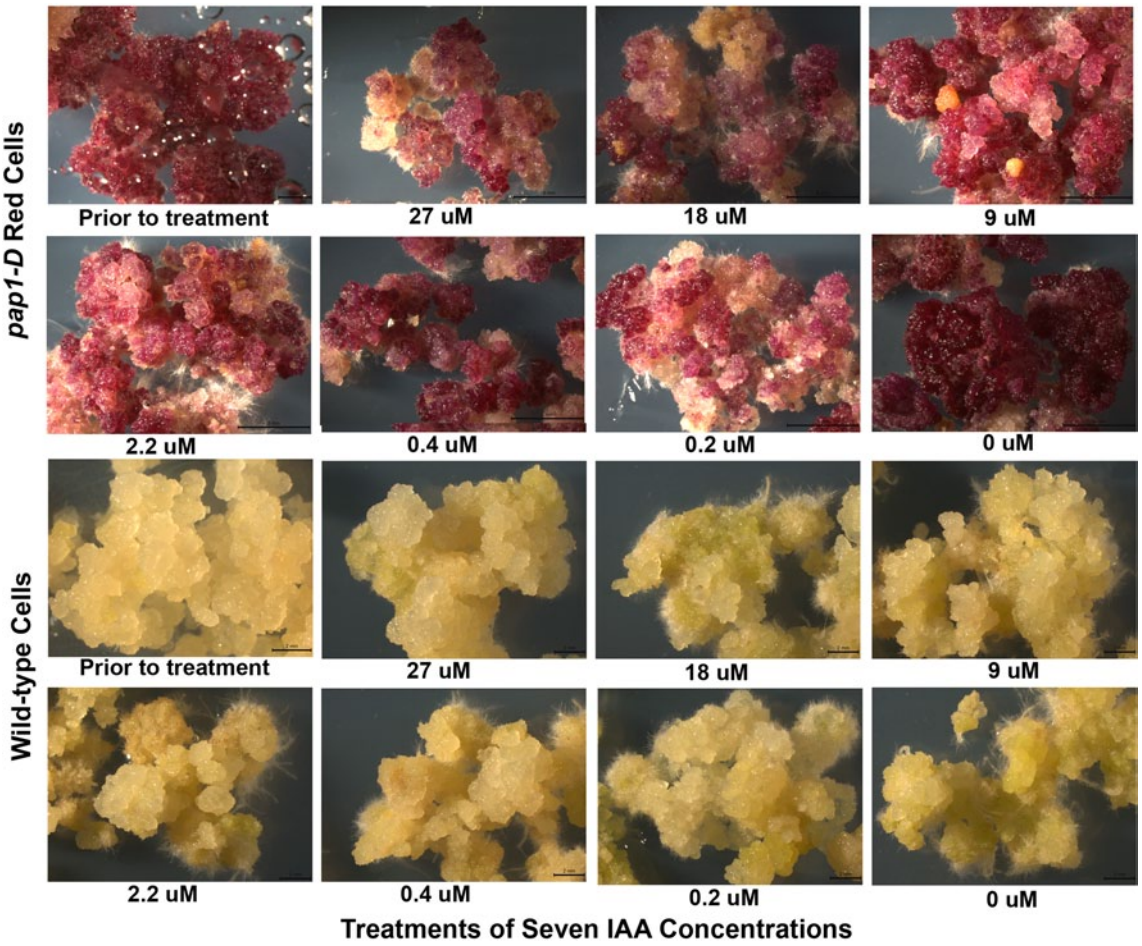

Fig. 4 The effects of seven different concentrations of IAA on the red pigmentation of calli

reduced. This result showed that the reduction of the pigmentation in the calli and the total anthocyanin production described above resulted from decreases in all of the seven anthocyanin molecules.

At the seven different concentrations of NAA, the chromatographs of the anthocyanins showed that the peak area and height values of the seven molecules were the highest at 9 μM NAA (Fig. 6). The area and height values of all seven anthocyanin peaks were reduced at NAA concentrations ranging from 0 to 0.4 μM, increased at NAA concentrations ranging from 0.4 to 9 μM, and then decreased again at NAA concentrations ranging from 9 to 27 μM. All peaks were simultaneously altered. This result supported the anthocyanin quantification data described above (Fig. 5b) and showed that alterations of total production resulted from changes of all seven anthocyanin molecules.

Of the seven concentrations of IAA, the peak area and height values of all seven anthocyanin molecules were the highest at 0 μM IAA. From 0.2 to 27 μM IAA, the levels of all the peaks were reduced, and the lowest peaks occurred at 27 μM IAA (Fig. 6). All peaks were simultaneously reduced. This result supported the pigmentation alterations (Fig. 4), the anthocyanin quantification data described above (Fig. 5c) and the decreases of production resulted from the reduction of all seven anthocyanin molecules.

Alterations in the expression of genes involved in anthocyanin biosynthesis upon 2,4-D treatment at various concentrations

The expression patterns of ten genes, *PAL1*, *CHS*, *DFR*, *ANS*, *PAP1*, *TT8*, *GL3*, *EGL3 MYBL2* and *TTG1,* were analyzed using semi-quantitative RT-PCR. Based on the band intensities of the amplified cDNA fragments on gels, the expression levels of the ten genes were characterized. The resulting data showed that red *pap1-D* cells treated with various concentrations of 2,4-D showed differences in the expression of nine of these genes (Fig. 7). *PAL1*, *CHS, DFR*, *ANS*, *PAP1* and *MYBL2* were expressed at an approximately similar level from 0 to 0.4 μM 2,4-D, at

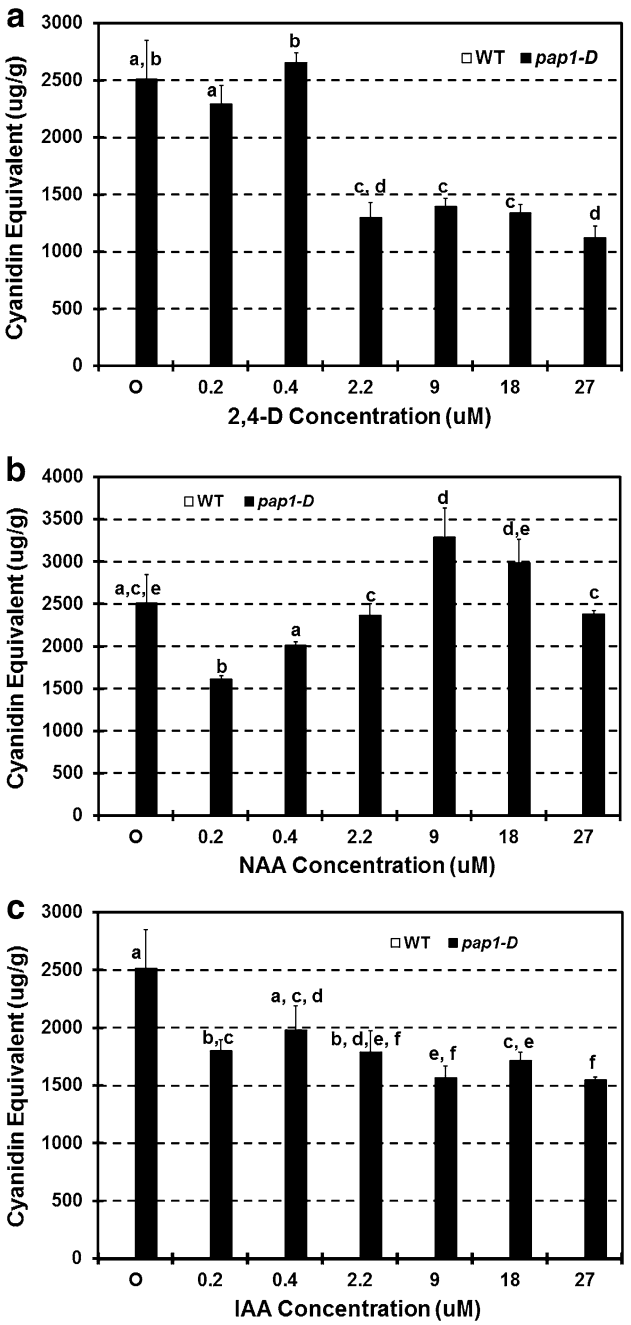

Fig. 5 The effects of various concentrations of 2,4-D (**a**), NAA (**b**), and IAA (**c**) on anthocyanin production. The columns labeled with different letters represent significantly different (*P* value <0.05) anthocyanin levels, while those with the same letter are not significantly different (*P* value >0.05). The anthocyanins were quantified using a wavelength of 530 nm on a UV spectrophotometer. Production values were indicted using a cyanidin equivalent

a reduced level at 2.2 μM 2,4-D and at a further reduced level at 2,4-D concentrations ranging from 9 to 27 μM. At 27 μM 2,4-D, the expression levels of these five genes were the lowest among all concentrations of 2,4-D tested. The expression of *TT8* and *GL3* increased from 0 to 0.4 μM 2,4-D and then decreased from 2.2 to 27 μM 2,4-D. Although *EGL3* was weakly expressed in red *pap1-D* cells, its expression levels increased slightly from 0 to 0.4 μM 2,4-D and then gradually decreased from 0.4 to 27 μM 2,4-D. In contrast to the other genes tested, the expression level of *TTG1* did not change significantly upon 2,4-D treatments.

Of the ten genes tested, the expression of six of them, *PAL1*, *CHS*, *TT8*, *GL3*, *EGL3* and *TTG1*, was detected in wild-type cells. The expression patterns of three of these genes (*CHS*, *GL3* and *EGL3*) were altered upon 2,4-D treatment. An opposite *CHS* expression pattern was revealed in wild-type cells compared to the red *pap1-D* cells. In the wild-type cells, the expression of this gene was barely detectable at 0 μM 2,4-D, but increased continuously from 0.2 to 2.2 μM 2,4-D and then remained at this level from 9 to 27 μM 2,4-D (Fig. 7). The expression level of *GL3* was similar from 0 to 0.4 μM 2,4-D and then decreased from 2.2 to 27 μM 2,4-D. As observed in red *pap1-D* cells, *EGL3* was weakly expressed in wild-type cells and its expression level was higher at 0 μM 2,4-D than at other concentrations. As observed in red *pap1-D* cells, the expression level of *TTG1* was similar in wild-type calli regardless of 2,4-D treatment. In addition, unlike in red *pap1-D* cells, the expression of *PAL1* in the wild-type cells was constant regardless of 2,4-D treatments. However, the expression of four genes, *DFR*, *ANS*, *PAP1*, and *MYBL2*, was not detected in wild-type cells in any of the conditions tested (Fig. 7), which was the cause leading to the lack of anthocyanin biosynthesis (Figs. 2, 5a, S-Fig. 2).

Alterations in the expression of genes involved in anthocyanin biosynthesis upon NAA treatment at various concentrations

The expression levels of *PAL1*, *CHS*, *DFR*, *ANS*, *PAP1*, *TT8*, *GL3*, *EGL3*, *TTG1* and *MYBL2* in red and wild-type calli treated with seven NAA concentrations were analyzed using semi-quantitative RT-PCR. Based on the band intensities of the RT-PCR products on agarose gels, the red *pap1-D* cells expressed all of these genes, and nine of them were altered by NAA (Fig. 8). The expression of *CHS*, *DFR*, *ANS*, *TT8* and *MYBL2* decreased from 0 to 0.2 μM NAA, stayed at a similar level from 0.2 to 2.2 μM NAA, increased at 9 μM NAA and then gradually decreased from 18 to 27 μM NAA. The expression levels of *GL3* and *EGL3* in red *pap1-D* cells were higher at 9 and 18 μM NAA than at other concentrations. The expression levels of *PAP1* were lower at 0 and 27 μM NAA than at other concentrations. In addition, the expression level of *PAL1* was lower at 27 μM NAA than at other concentrations. Unlike the other genes, the expression of *TTG1*

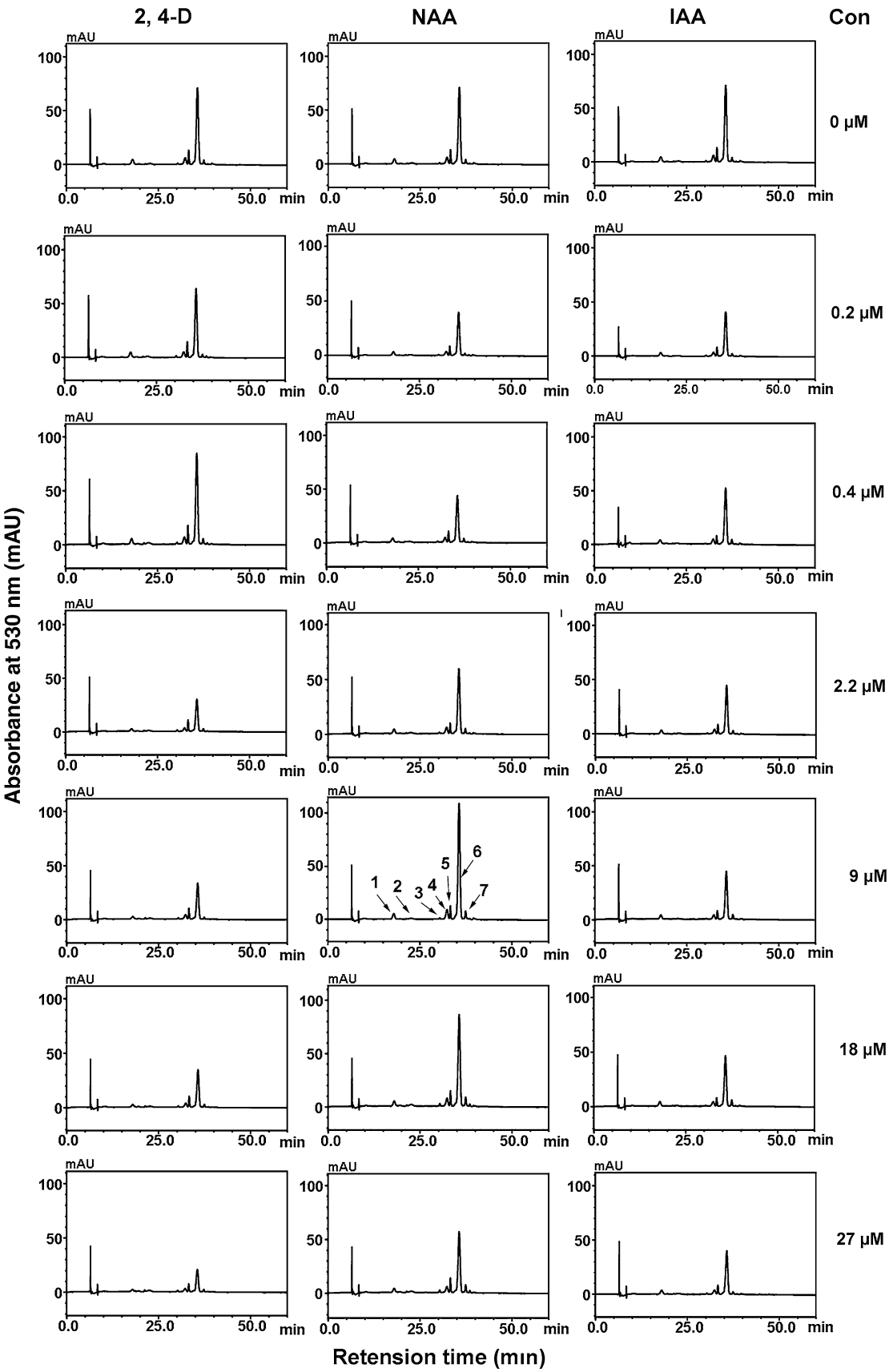

Fig. 6 The effects of various concentrations of 2,4-D, NAA and IAA on the anthocyanin profile. The chromatographs of anthocyanins were recorded at 530 nm

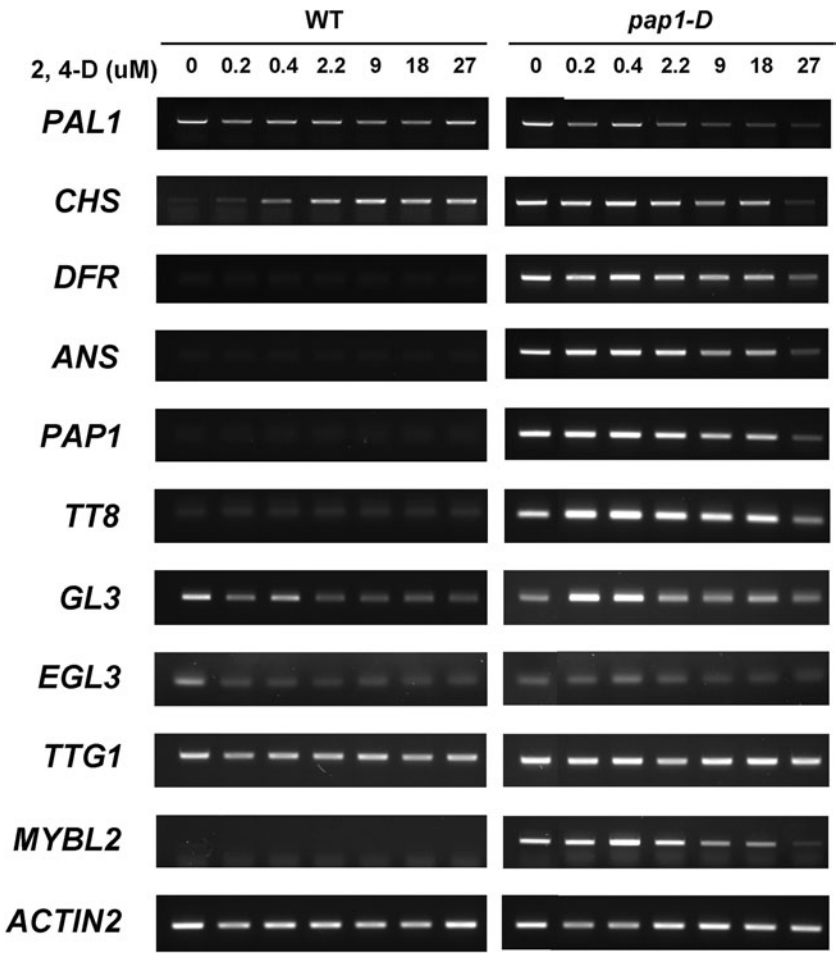

Fig. 7 RT-PCR analysis showing the transcript levels of ten genes involved in anthocyanin biosynthesis in red *pap1-D* cells and wild-type treated with seven different concentrations of 2,4-D. RT-PCR was performed using gene-specific primers to analyze transcripts of four pathway and six regulatory genes in red *pap1-D* vs. wild-type cells. The four biosynthetic pathway genes are *PAL1* phenylalanine ammonialyse 1, *CHS* chalcone synthase, *DFR* dihydroflavonol reductase, *ANS* anthocyanidin synthase. The six regulatory genes are *EGL3* enhancer glabra 3, *GL3* glabra 3, *MYBL2* (Arabidopsis MYB-like 2), *PAP1* production of anthocyanin pigmentation 1, *TT8* transparent testa 8, *TTG1* transparent glabra 1

did not change evidently in red *pap1-D* cells upon NAA treatments.

In wild-type cells, RT-PCR analysis showed cDNA products for only five of the genes tested, *PAL1*, *CHS*, *GL3*, *EGL3* and *TTG1* (Fig. 8). The *PAL1* expression pattern was similar in wild-type and red *pap1-D* cells; its expression level at 27 μM NAA was lower than at other concentrations of NAA. *CHS* expression was barely detectable at 0–9 μM NAA and moderately increased at 18 and 27 μM NAA. The changes in *GL3* expression upon NAA treatment were similar in wild-type and red *pap1-D* cells, although *GL3* was expressed more highly in the wild-type cells than in the red *pap1-D* cells. Similarly, *EGL3* expression levels were higher in the wild-type cells than in the red *pap1-D* cells at some concentrations of NAA. In the wild-type cells, *EGL3* decreased from 0 to 0.2 μM NAA, slightly increased from 0.2 to 2.2 μM NAA, remained at a similar level from 2.2 to 18 μM NAA and decreased at 27 μM NAA, which was different from the expression pattern in red *pap1-D* cells described above (Fig. 8). *TTG1* expression did not change upon NAA treatment. However, *DFR*, *ANS*, *PAP1* and *MYBL2* expression was not detected in wild-type cells (Fig. 8), which caused the lack of anthocyanin biosynthesis (Figs. 2, 5b, S-Fig. 2).

Alterations in the expression patterns of genes involved in anthocyanin biosynthesis upon IAA treatment at various concentrations

RT-PCR analysis showed that red *pap1-D* cells expressed *PAL1*, *CHS*, *DFR*, *ANS*, *PAP1*, *TT8*, *GL3*, *EGL3*, *TTG1* and *MYBL2* at all seven concentrations of IAA (Fig. 9). The expression patterns of nine of these genes were affected by IAA treatment (Fig. 9). From the gel images, it was apparent that as the concentration of IAA increased, levels of *CHS*, *DFR*, *ANS*, *PAP1* and *MYBL2* gradually and slightly decreased. *GL3* and *EGL3* levels were reduced from 0 to 0.2 μM IAA treatment, increased at 0.4 μM IAA and then decreased from 2.2 to 27 μM IAA. At 27 μM IAA, the intensity of these two gene fragment bands was the weakest. The expression level of *TT8* was constant at 0–18 μM IAA, but was obviously reduced at 27 μM IAA. Unlike the other genes, *TTG1* and *PAL1* expression levels were not altered by IAA.

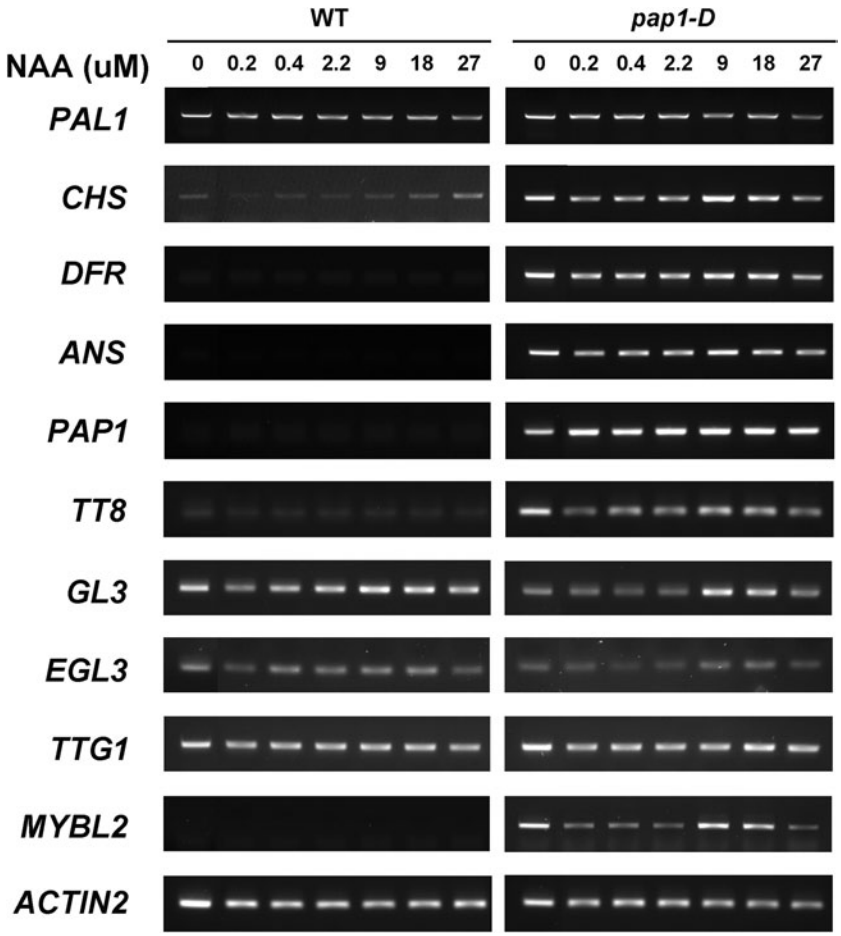

Fig. 8 RT-PCR analysis showing the transcript levels of ten genes involved in anthocyanin biosynthesis in red *pap1-D* cells and wild-type treated with seven different concentrations of NAA. RT-PCR was performed using gene-specific primers to analyze transcripts of four pathway and six regulatory genes in red *pap1-D* vs. wild-type cells. The four pathway genes are *PAL1* phenylalanine ammonialyse 1, *CHS* chalcone synthase, *DFR* dihydroflavonol reductase, *ANS* anthocyanidin synthase. The six regulatory genes are *EGL3* enhancer glabra 3, *GL3* glabra 3, *MYBL2* (Arabidopsis MYB-like 2), *PAP1* production of anthocyanin pigmentation 1, *TT8* transparent testa 8, *TTG1* transparent glabra 1

RT-PCR analysis showed that the wild-type cells expressed *PAL1*, *CHS*, *GL3*, *EGL3* and *TTG1* (Fig. 9). In contrast, the expression of *TT8* and *MYBL2* was barely detectable. Images of gels showed that the intensities of *PAL1* cDNA fragments amplified by RT-PCR exhibited a slight increase from 0 to 27 μM IAA. *GL3* was highly expressed in wild-type cells, and its expression was slightly altered by IAA. Its expression decreased slightly from 0 to 0.2 μM IAA, increased at 0.4 μM IAA and slightly increased from 2.2 to 27. At 27 μM IAA, the intensity of the amplified cDNA band was the brightest. The expression of *GL3* at most IAA concentrations in wild-type cells was higher than in red *pap1-D* cells (Fig. 9). *EGL3* was expressed in wild-type cells at all tested IAA concentrations. Compared to those for *GL3*, the intensities of the cDNA bands for *EGL3* were much weaker, although the *EGL3* and *GL3* expression patterns were similar. *CHS* expression at all IAA concentrations was barely detectable in wild-type cells and much lower than in red *pap1-D* cells. In contrast, *TTG1* was not altered by IAA. The expression levels of *DFR*, *ANS* and *PAP1* were undetectable in wild-type cells under all seven conditions (Fig. 9). The lack of *DFR*, *ANS* and *PAP1* expression and the low *CHS* and *TT8* expression prevented anthocyanin biosynthesis in the wild-type cells (Figs. 4, 5c, and S-Fig. 2).

Discussion

The data from 42 treatments (21 for the red *pap1-D* cells and 21 for the wild-type control cells) demonstrated that anthocyanin biosynthesis in the red *pap1-D* cells is controlled by genes encoding the TTG1-TT8/GL3-PAP1 complex, as previously reported (Xie and Shi 2012; Zhou et al. 2012; Shi and Xie 2011). In the 21 auxin treatments, the red *pap1-D* cells, but not the wild-type cells, produced anthocyanins (Figs. 2, 3, 4, 5). RT-PCR analysis demonstrated a correlation between the expression of *TT8*/*GL3* and *PAP1* and anthocyanin biosynthesis in the red *pap1-D* cells. In contrast, *PAP1* is not expressed in wild-type cells (Figs. 7, 8, 9). The expression of *TT8* is also barely detected in wild-type cells (Figs. 7, 8, 9). *TTG1* appears to be constitutively expressed, and *GL3* is also expressed in wild-type cells. Our data demonstrate that without *PAP1* and *TT8* expression, the expression of *TTG1* and *GL3* is

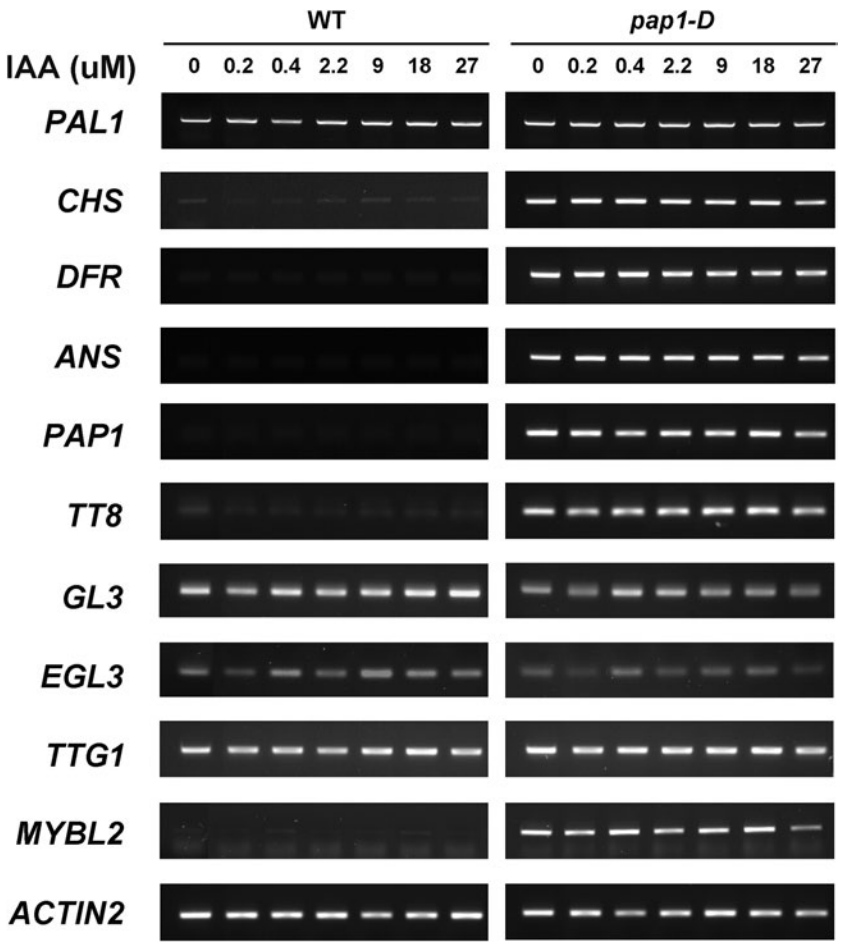

Fig. 9 RT-PCR analysis showing the transcript levels of ten genes involved in anthocyanin biosynthesis in red *pap1-D* and wild-type cells treated with seven different concentrations of IAA. RT-PCR was performed using gene-specific primers to analyze transcripts of four pathway and six regulatory genes in red *pap1-D* vs. wild-type cells. The four pathway genes are *PAL1* phenylalanine ammonialyse 1, *CHS* chalcone synthase, *DFR* dihydroflavonol reductase, *ANS* anthocyanidin synthase. The six regulatory genes are *EGL3* enhancer glabra 3, *GL3* glabra 3, *MYBL2* (Arabidopsis MYB-like 2), *PAP1* production of anthocyanin pigmentation 1, *TT8* transparent testa 8, *TTG1* transparent glabra 1

insufficient in forming a complete complex that activates anthocyanin biosynthesis.

NAA, 2,4-D and IAA control anthocyanin biosynthesis through regulating the expression of genes encoding transcription factors to form the TTG1-TT8/GL3-PAP1 complex in red *pap1-D* cells. 2,4-D exhibited a strong inhibitory effect on anthocyanin production in the tested concentration range of 2.2–27 μM (Figs. 2, 5a). In NAA treatments, compared to control (0 μM NAA), NAA negatively regulates anthocyanin biosynthesis at 0.2 μM and then promotes it at 0.4–9 μM with the maximum effect occurring at 9 μM. This is followed by a reduction in anthocyanin biosynthesis at the two higher concentrations (Fig. 5b). Compared to control (0 μM), IAA concentrations from 0.2 to 27 μM used in media lead to reduction of anthocyanin levels (Figs. 4, 5c). Although the effects of the three auxins on anthocyanin biosynthesis are different, our data showed that the regulation of anthocyanin biosynthesis by these three auxins resulted from altered expression levels of the regulatory complex genes that govern the expression of biosynthetic pathway genes. Here, we suggest that 2,4-D may perturb the formation of the TTG1-TT8/GL3-PAP1 (WBM) complex, thus resulting in a reduction in the transcription levels of biosynthetic pathway genes. RT-PCR analysis revealed that in all 2,4-D treatments, anthocyanin production was tightly associated with the expression levels of some tested candidate genes, including *PAP1*, *TT8*, and *GL3* (Fig. 7). These data allow us to propose that the reductions in *PAP1* and *TT8*/*GL3* expression most likely lead to a decrease in their encoded products, MYB75 and two bHLH proteins, respectively. As reported in the literature, TTG1, TT8/GL3 and PAP1 form a WD40-bHLH-MYB complex (Gonzalez et al. 2008; Xie and Shi 2012). Therefore, we further hypothesized that in the range of concentrations of 2.2–27 μM of 2,4-D, red *pap1-D* cells may not form sufficient WD40-bHLH-MYB (WBM) complexes. To demonstrate this, RT-PCR analysis was performed on red *pap1-D* and wild-type cells. The resulting data showed that the expression levels of representative early and late biosynthetic pathway genes (*CHS*, *DFR* and *ANS*) gradually decreased as the concentration of 2,4-D increased from 0.4 to 27 μM (Fig. 7). In addition, the level of *PAL1* expression clearly decreased at high concentrations of 2,4-D. Although our previous transcriptomic experiments showed insignificant difference in the expression level of *PAL1* between red *pap1-D* and wild-type cells at 0.47 μM 2,4-D (Shi and

Xie 2011), here we observed that high concentrations of 2,4-D strongly inhibited *PAL1* expression in the red *pap1-D* cells, but not in the wild-type cells (Fig. 7). Based on this interesting observation, we hypothesize that the TTG1-TT8/GL3-PAP1 complex may conditionally participate in the regulation of *PAL1* in red *pap1-D* cells. This phenomenon is open for future researches. In addition, it is interesting that opposite expression profiles of *CHS* were observed between red *pap1-D* and wild-type cells in all seven treatments of 2,4-D (Fig. 7). This observation implies that *CHS* transcription is likely controlled by the TTG1-TT8/GL3-PAP1 complex along with other factors. In addition, IAA and NAA appear to control anthocyanin biosynthesis similarly to 2,4-D, as described above. These two auxins alter the expression of genes encoding transcription factors to form the TTG1-TT8/GL3-PAP1 complex and the expression of biosynthetic pathway genes. Our data show that the production of anthocyanins (Fig. 5b, c) is tightly associated with the expression levels of genes including *PAP1*, *TT8*, *GL3*, *CHS*, *DFR* and *ANS* tested in our experiments (Figs. 8, 9). It is interesting that the expression level of *GL3* is generally lower in red *pap1-D* cells than in wild-type cells upon treatment with NAA and IAA (Figs. 8, 9), but not with 2,4-D (Fig. 7). The reason for this difference is unknown. We suggest that this phenomenon is likely due to pleiotropic activities of GL3. As well understood, in addition to its involvement in anthocyanin biosynthesis, GL3 is essentially involved in pavement cell and trichome formation (Payne et al. 2000; Bernhardt et al. 2003, 2005; Zhao et al. 2008; Feyissa et al. 2009). In our experiments on media containing NAA or IAA, but not media containing 2,4-D, the wild-type and the red *pap1-D* calli exhibited an increased number of root hairs (Figs. 2, 3, 4). Based on these observations, we hypothesize that the induction of root hairs by IAA and NAA is associated with GL3. The mechanisms involved in the different responses of *GL3* expression to various auxins will be investigated in future studies. Furthermore, we suggest that the red *pap1-D* cells can enhance the understanding of anthocyanin biosynthesis in other plant cell cultures. In vitro engineering of anthocyanin biosynthesis has been investigated for several decades in different callus and suspension culture systems (Ball 1967; Stickland and Sunderland 1972; Wellmann et al. 1976; Dougall et al. 1980; Al Qurraan et al. 2012; Asano and Otobe 2011). Examples of long-term model callus and cell suspension cultures are carrots (Dougall et al. 1980, 1983; Rose et al. 1996; Hirner and Seitz 2000; Narayan et al. 2005; Ozeki et al. 2000), grapes (Bao Do and Cormier 1991; Do and Cormier 1991a, b; Cormier et al. 1992; Yousef et al. 2004) and *Catharanthus roseus* (Hall and Yeoman 1986a, b). Numerous investigations have provided evidence regarding regulation of anthocyanin biosynthesis in vitro, including the identifications of the anthocyanin molecules, enzymes and genes. In all callus and cell suspension cultures, the optimization of auxins and other phytohormones is an essential step to induce the formation of anthocyanins. Numerous experiments have shown that the removal of 2,4-D from the media or a low concentration of 2,4-D is necessary for the induction of anthocyanins (Ozeki and Komamine 1985, 1986; Yoshihiro and Atsushi 1985; Ozeki et al. 1990). 2,4-D down-regulates gene expression, leading to reductions in pathway enzymes, such as PAL and CHS, in carrot cell and callus cultures (Ozeki et al. 1990, 2000). However, the regulatory mechanisms in these in vitro systems remain largely unknown. Our data provide molecular evidence that can enhance the understanding of theregulatory mechanisms controlling anthocyanin biosynthesis in tissue and cell cultures of other plants. In addition, due to key roles of auxins in plant growth and development, we hypothesize that auxins may alter gene expressions involved in other metabolisms directly or indirectly impacting anthocyanin biosynthesis in red cells. To date, these kinds of researches are highly lacking. The red vs. wild-type cell cultures under this report form an appropriate model system to understand the effects of auxins on genome-wide metabolisms.

MYBL2 (a R3-MYB member) is a negative regulator of anthocyanin biosynthesis in seedlings (Matsui et al. 2008; Dubos et al. 2008). Mutations in this gene activate anthocyanin biosynthesis, and thus, it is defined as a transcriptional repressor. Furthermore, its repression activity has been demonstrated to result from its direct binding to TT8, leading to reductions in *DFR* and *ANS* transcription (Matsui et al. 2008). However, in our previous study, we showed that under various nitrogen treatments, *MYBL2* was expressed in red *pap1-D* cells, but not in wild-type cells, which lack anthocyanins. Furthermore, *MYBL2* expression positively correlates with anthocyanin production (Zhou et al. 2012). In our present study, we observed that in all 27 auxin treatments, the expression of *MYBL2* was detected in red *pap1-D* cells, but not detected or only barely detected in wild-type cells (Figs. 7, 8, 9). *MYBL2* expression also positively correlated with *DFR*, *ANS*, *PAP1* and *TT8* transcription. These data do not show the repression by *MYBL2* on *DFR* and *ANS* transcription. Therefore, we suggest that the repression of anthocyanin biosynthesis by MYBL2 is conditional.

Our data enhance our understanding of the involvement of auxins in plant secondary metabolism and may guide agricultural efforts. Auxin is essentially involved in all plant growth and development processes, and the mechanisms behind those processes have gained much attention from researchers in the fields of genetics, molecular biology, and functional genomics (Yoshida et al. 2013; Strader and Nemhauser 2013; Rahman 2013). In comparison, the effects of auxin on plant secondary metabolism remain

largely cryptic, although knowledge in this area is of great agricultural and horticultural significance. As we know, two common natural auxins exist, IAA (indole-3 acetic acid) and IBA (indole-butyric acid), the latter of which has been proposed to be a precursor of the former (Strader and Nemhauser 2013). Five common synthetic auxin compounds are 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA), 2-methoxy-3,6-dichlorobenzoic acid (MDBA), 4-amino-3,5,6-trichloropicolinic acid (ATPA), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). These compounds have been applied in horticultural studies to regulate fruit and tuber development as well as anthocyanin formation. Examples of the fruits and tubers studied in this manner include potatoes, grapes and apples. In one study, treating red Norland potatoes with 2,4-D led to an increase in the ratio of peonidin derivatives to pelargonidin derivatives, two anthocyanin metabolites. When developing grape berries were treated with NAA prior to veraison, fruit ripening and anthocyanin formation were delayed (Bottcher et al. 2011). In addition to anthocyanin profiles, volatiles and other metabolites were also altered in the delayed-ripening berries (Bottcher et al. 2011, 2012). When 100 mg/l of 2,3,5-triiodobenzoic acid (TIBA) was applied to grapes at the beginning of veraison, the anthocyanin content increased significantly in the skins of the ripened fruits (Yakushiji et al. 2001). When 50 mg/l 2,4-dichlorophenoxypropionic acid (2,4-DP) was applied to developing apples, the anthocyanin pigmentation increased in the skin (Stern et al. 2010). In addition, 2,4-D esters and amines were shown to strongly impact tuber color (Rosen et al. 2009). Mesocarp discs of peach fruits treated with NAA (up to 100 μM) had significantly increased levels of anthocyanins under light condition (Ohmiya 2000). These studies demonstrated that exogenous synthetic auxins alter anthocyanin biosynthesis. Based on our experiments under this report, we hypothesize that these auxins alter anthocyanin biosynthesis via a mechanism involving transcription factor complexes.

Our data are valuable for the metabolic engineering of other medicinal metabolites. Cell and callus cultures are promising systems for understanding the biosynthesis of plant natural products (Parr 1989). For example, vinca alkaloids, including vinblastine, vincristine, vindesine, and vinorelbine produced by *C. roseus*, are important anti-leukemia medicines (Mukherjee et al. 2001; St-Pierre and De Luca 1995). Many experiments using callus or cell suspension cultures have been performed to understand biosynthesis for the purpose of metabolic engineering these anti-cancer compounds (Kurz et al. 1980; Kutney et al. 1980; Spitsberg et al. 1981; Grabowski et al. 1991; Decendit et al. 1992). The data from these investigations demonstrated that auxins negatively regulate biosynthesis of vinca alkaloids. When auxins are removed from culture media, the production of vinca alkaloids such as ajmalicine is increased in cultured *C. roseus* cells (Decendit et al. 1992). Strictosidine synthase (SSS) catalyzes the formation of strictosidine, a key intermediate in vinca alkaloid production. In a report on suspension cell cultures, auxins were determined to down-regulate the expression of *SSS* (Pasquali et al. 1992). A recent investigation demonstrated that the regulation by auxin on vinca alkaloid biosynthesis occurs via an interaction with an Aux/IAA protein (Poutrain et al. 2011). On the one hand, these experiments have provided fundamental information for improving metabolic engineering of vinca alkaloids. On the other hand, they indicate that the mechanism of regulation by auxin on vinca alkaloid formation remains elusive. Furthermore, there are many medicinal metabolites, such as quinine, morphine and codeine, the biosynthesis of which remains to be studied. Quinine is an anti-malarial quinoline alkaloid compound synthesized by *Cinchona ledgeriana*. In tissue cultures of this anti-malarial plant, the removal of 2,4-D by low concentrations of NAA is an essential step in increasing quinine production (Hoekstra et al. 1990). In tissue cultures of *Papaver somniferum*, the absence of exogenous auxins and cytokinins in the medium has been shown to increase the production of both morphine and codeine (Siah and Doran 1991). These experiments imply that the auxins used in the culture medium regulate the biosynthesis of many plant natural products. Although the understanding of the biosynthetic pathways and the regulatory mechanisms of these metabolites is limited because the genomes of these three medicinal plants are not fully sequenced, it can be expected that as more genes involved in the biosynthesis of plant natural products are identified, the biosynthetic regulatory mechanisms will be elucidated. Understanding the biosynthetic pathways of these natural products and their regulation will improve metabolic engineering.

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