Plant Physiology and Biochemistry 142 (2019) 332–341

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Application of melatonin promotes anthocyanin accumulation in crabapple leaves

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ARTICLE INFO

Keywords:
Melatonin
Anthocyanins
Malus crabapple
Leaves
Flavonols
Proanthocyanins

ABSTRACT

Anthocyanins are a class of compounds that are widespread in plants, where they provide protection against stresses, and are also beneficial to human health as dietary components. Melatonin application is known to affect anthocyanin production, but the relationship between anthocyanin and melatonin is still unclear. In this study, we analyzed anthocyanin contents and the expression levels of anthocyanin biosynthetic and regulatory genes in tissue cultured plantlets of two *Malus* crabapple cultivars following various exogenous melatonin treatments under light and dark conditions. The application of exogenous melatonin not only promoted anthocyanin accumulation in leaves, but also increased the contents of flavonols and proanthocyanins (PAs), via a process that was not dependent on light. Quantitative real time PCR (qRT-PCR) analyses indicated that the expression of flavonoid biosynthetic genes, flavonoid related transcription factors and melatonin biosynthetic genes was induced by melatonin. We propose that anthocyanin biosynthesis is regulated by melatonin in crabapple leaves via the expression of flavonoid related transcription factors. This study provides insight into the mechanism of melatonin induction of anthocyanin biosynthesis in woody plants, and suggests that pretreatment with melatonin may represent a cultivation strategy to increase the flavonoid contents of plants.

1. Introduction

Anthocyanins are pigments that are responsible for the red, purple and blue coloration in a wide range of plant tissues and organs, but are particularly prevalent in the fruits, leaves and flowers of ornamental crops (Koes et al., 2005; Grotewold, 2006; Lepiniec et al., 2006; Gould, 2007). They have multiple roles, including promoting tolerance of environmental stresses, enhancing resistance to herbivores and pathogens, attracting pollinators and seed dispersers, and functioning as antioxidants and free radical scavengers. Consequently, they both protect plants and are beneficial to human and animal health as dietary components (Harrison and Stickland, 1974; Pourcel et al., 2007; Butelli et al., 2008; Gonzali et al., 2009).

Anthocyanins are synthesized through the flavonoid pathway, and anthocyanin biosynthetic genes from many plant species have been characterized, including phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), dihydroflavonol 4-reductase (DFR), anthocyanin synthase (LDOX) and UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT) (Winkel-shirley, 2001). Their functions have been correlated with anthocyanin accumulation in a range of plant species and tissues, such as the expression of most anthocyanin biosynthetic genes in leaves, petals and fruit skin in fruit of the *Malus* species, apple (*Malus domestica*) and *Malus* crabapple (Honda et al., 2002; Espley et al., 2007; Jiang et al., 2014; Tian et al., 2015b).

The expression of anthocyanin biosynthetic genes is coordinately modulated by termed the MBW complex, consisting of MYB transcription factors, basic helix-loop-helix (bHLH) transcription factors, and WD40 proteins (Ramsay and Glover, 2005; Saito et al., 2013). The regulatory mechanism of bHLH and MYB transcription factors has been intensively studied in model plants, including *Arabidopsis thaliana*, *Petunia* and tomato (*Solanum lycopersicum*) (Ramsay and Glover, 2005; Feng et al., 2010; Sullivan et al., 2003; Arnao and Hernándezruiz, 2010a, 2010b); and in crop plants, including apple, pear (*Pyrus*

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https://doi.org/10.1016/j.plaphy.2019.07.024
Received 4 April 2019; Received in revised form 29 July 2019; Accepted 29 July 2019
Available online 30 July 2019
0981-9428/

pyrifolia), peach (*Prunus persica*) and mangosteen (*Garcinia mangostana* L.) (Espley et al., 2007; Nawaz et al., 2016; Arnao and Hernández-Ruiz, 2017; Palapol et al., 2009). In *Malus* species, MdMYB1, MdMYB10, MdMYBA and McMYB10 have been shown to interact with bHLH3 and WD40 proteins to regulate the transcript abundance of anthocyanin biosynthetic genes, thereby controlling anthocyanin accumulation and pigmentation (Espley et al., 2007; Jiang et al., 2014; Tian et al., 2015b; Palapol et al., 2009; Ban et al., 2007).

It has been well documented that anthocyanin accumulation may be induced by sugars and hormones, as well as various environmental factors, including light, temperature and soil pH value (Takos et al., 2006; Wan et al., 2015; Zhang et al., 2014; Xie et al., 2012). Many studies have indicated that the expression of bHLH and MYB transcription factors, which are members of the MBW complex, is induced or repressed by environmental factors and other stimuli (Ban et al., 2007; Rowan et al., 2010; Aharoni et al., 2001). Thus, anthocyanin biosynthetic genes are regulated by the up- or down regulation of bHLH and MYB transcription factors, which results in altered anthocyanin accumulation and pigmentation (Ban et al., 2007; Wan et al., 2015; Matoušek et al., 2012).

Melatonin and anthocyanins are known to have similar roles as antioxidants in plants; however, little is known about the relationship between the two. Melatonin (N-acetyl-5-methoxytryptamine) is ubiquitous in mammals, bacteria, fungi, and many plant species (Hernándezruiz et al., 2004; Hernández-Ruiz et al., 2010; Hernándezruiz and Arnao, 2008a,b; Chen et al., 2009; Posmyk et al., 2009; Zhang et al., 2012; Sarropoulou et al., 2012a,b; Park and Back, 2012; Pelagio-Flores et al., 2012), controlling circadian rhythms and photoperiodic reactions (Kolář et al., 2003; Zhao et al., 2013), delaying senescence by regulating photosynthetic systems and protecting chlorophyll (Tettamanti et al., 2000; Paredes et al., 2009), as well as promoting tolerance of cold and drought (Bajwa et al., 2014; Shi and Chan, 2015; Chen et al., 2018; Zuo et al., 2015; Zhang et al., 2013; Arnao and Hernándezruiz, 2010a, 2010b). Melatonin also acts as an antioxidant.

Malus crabapple varieties, which belong to the Rosaceae, *Malus Mill* family, show a diverse range of leaf, flower and fruit colors, and are represented by an economically important germplasm collection (Jiang et al., 2014; Tian et al., 2017). The objective of the current study was to evaluate how anthocyanins in crabapple leaves respond to a melatonin treatment, which has previously been shown to promote anthocyanin production in other species, such as tomato (*Solanum lycopersicum* cv. Bmei) (Sun et al., 2016). Specifically, we applied different concentrations of melatonin to the ever-red crabapple cultivar 'Royalty' and the spring-red cultivar 'Radiant'. Based on high pressure liquid chromatography (HPLC) analysis of flavonoid components and expression assays of anthocyanin biosynthetic genes, we propose that melatonin plays an important role in altering anthocyanin accumulation and leaf pigmentation.

2. Materials and methods

2.1. Plant materials and growth conditions

Explants of *Malus* cv. 'Royalty' (the color of both young and mature leaves ranges from red to purple) and 'Radiant' (young leaves are red and mature leaves are green) were harvested from one-year-old branches before spring bud germination and cultured on Murashige and Skoog (MS) medium supplemented with 1 mg/L 6-benzylaminopurine (6-BA) and 2 mg/L 1-naphthylacetic acid (NAA) at 22 °C with a 16 h light (200 μmoL. S^{-1}. M^{-2})/8 h dark period for 30 d. Tissue cultured plantlets were preserved at the tissue culture center of Beijing University of Agriculture.

2.2. Melatonin treatments

Melatonin with various concentrations (0, 50, 100, 200, 500, 1000 μg/L) (Sigma-Aldrich, St. Louis, MO, USA) were filter-sterilized and added to the cooled above described MS medium (55°C) under low light. 30-day-old explants transferred to these mediums to test the effects of exogenous melatonin. Explants placed on MS medium with 0 mg/L melatonin were used as controls. A total of 50 explants for each treatment were placed in the light or dark. The new buds of treated explants were frozen in liquid nitrogen upon collection and stored at −80 °C prior to HPLC analysis or RNA extraction.

2.3. HPLC analysis

Frozen crabapple leaf samples (approximately 0.8–1.0 g fresh weight) were ground in 10 mL extraction solution (methanol: water: formic acid: trifluoroacetic acid = 70: 27: 2: 1) and placed at 4 °C in the dark for 72 h, shaking every 6 h. The supernatant was then passed through filter paper and then through a 0.22 μm Millipore™ filter (Billerica, MA, USA). For the HPLC analysis, trifluoroacetic acid: formic acid: water (0.1: 2: 97.9) was used as mobile phase A and trifluoroacetic acid: formic acid: acetonitrile: water (0.1: 2: 48: 49.9) was used as mobile phase B. The gradients used were as follows: 0 min, 30% B; 10 min, 40% B; 50 min, 55% B; 70 min, 60% B; 30 min, 80% B. Detection was performed at 520 nm for Cyanidin-3,5-diglucoside and 280 nm for Pas, The unit is mg kg^{-1} (Tian et al., 2017). All samples were analyzed in three biological triplicates.

2.4. Quantitative real time PCR (qRT-PCR) analysis

Total RNA was extracted from crabapple leaves using an RNA Extraction Kit (Aidlab, Beijing, China) according to the manufacturer's instructions. DNase I (TaKaRa, Ohtsu, Japan) was added to remove genomic DNA, and the samples converted to cDNA using the Access RT-PCR System (Promega, USA), according to the manufacturer's instructions. The expression levels of flavonoid biosynthetic and regulatory genes were analyzed using qRT-PCR and the SYBR Green qPCR Mix (TaKaRa, Ohtsu, Japan) with the Bio-Rad CFX96 Real-Time PCR System (BIO-RAD, USA), according to the manufacturers' instructions. The gene sequences of melatonin biosynthetic enzymes (MdTDC, MdT5H, MdAANAT, and MdASMT) were obtained from the *M. domestica* Genome database (http://genomics.research.iasma.it/gb2/gbrowse/apple/). The PCR primers were designed using NCBI Primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Table 1 qRT-PCR analysis was carried out in a total volume of 20 μl containing 9 μl of 2 × SYBR Green qPCR Mix (TaKaRa, Ohtsu, Japan), 0.1 μM specific primers (each), and 100 ng of template cDNA. The reaction mixtures were heated to 95 °C for 30 s, followed by 39 cycles at 95 °C for 10 s, 50–59 °C for 15 s, and 72 °C for 30 s. A melting curve was generated for each sample at the end of each run to ensure the purity of the amplified products. The transcript levels were normalized using the *Malus* 18S ribosomal RNA gene (GenBank ID DQ341382) as the internal control and calculated using the $2^{\wedge(-\Delta\Delta Ct)}$ analysis method (Zhao et al., 2013).

2.5. Data analysis

All data were analyzed using one-way ANOVA followed by Duncan's SSR test (shortest significant ranges) to compare differences among the experimental sites at $P < 0.05$ (Microsoft Excel 2003 and Data Processing System (DPS) software 7.05).

2.6. Accession number

McCHS (FJ599763), McF3H (FJ817486), McF3'H (KF481684), McDFR (FJ817487), McANS (FJ817488), McUFGT (KF495603), McANR1 (KT276930), McANR2 (KT276931), McLAR1 (KT276929), McMYB4 (JX013493), McMYB10 (JX162681), McMYB12 (KJ020112), McbHLH3 (HM122458), McbHLH33 (DQ266451), 18S RNA

Table 1
The primers used for qRT-PCR.

Primers	Primer sequences
McCHS-F	5′- TGACCGTCGAAGTTCGC -3′
McCHS-R	5′- TTTGTCACACATGCGCTGGA -3′
McF3H-F	5′- ACGAAGACGAGCGTCCAAAG -3′
McF3H-R	5′- CTCCTCCGATGGCAAAGCAA -3′
McF3′H-F	5′- CGTTGCTGTCGCTCACGGATGA -3′
McF3′H-R	5′- ATGACGTGTCAGTGCCAGCTGTG -3′
McDFR-F	5′- CCGAGTCCGAATCCGTTTGT -3′
McDFR-R	5′- CCTTCTTCTGATTCGTGGGGT -3′
McANS-F	5′- CACAGGGGCATGGTGAACAA -3′
McANS-R	5′- TTCACTTGGGGAGCAAAGCC -3′
McUFGT-F	5′- TGGGCGGACACCAATCA -3′
McUFGT-R	5′- ATGTCTCCACCGCACCA -3′
McFLS-F	5′- ACGAGCAACCGGGAATCACAACTG -3′
McFLS-R	5′- CCCAGTTGGAGCTGGCCTCAGTA -3′
McANR1-F	5′- AACCACAAGAAGGTCTCCCAC -3′
McANR1-R	5′- CCCTTGGATTGCTGGTTTGAT -3′
McANR2-F	5′- ACCCCTGTCAACTTTGCCTCA -3′
McANR2-R	5′- CCAAACCTGTTCCCTCAAGTGTAT -3′
McLAR1-F	5′- TTTATCAAAGGATGCCAGGTT -3′
McLAR1-R	5′- CATCCAAGGTCCTGAAAGAAT -3′
McMYB4-F	5′- GACCAGCAGCAGAAACTA -3′
McMYB4-R	5′- ACAACCCTCCATTAATGCCGAC -3′
McMYB10-F	5′- ACGCCACCACAAACGTCGTCG -3′
McMYB10-R	5′- GGCGCATGATCTTGGCGACAGT -3′
McMYB12-F	5′- CAGCAAGTGCTAAGATGCAAAC -3′
McMYB12-R	5′- GCTATCAAAGACCACCGATTG -3′
McbHLH3-F	5′- CACTAACCAATCAAACCCAAATCCC -3′
McbHLH3-R	5′- AACCCTAAATGATCCTGCAGATTTCG -3′
McbHLH33-F	5′- GGAAAATGGCTCAGAATCAT -3′
McbHLH33-R	5′- CTCAGCACTTACCAGCAATT -3′
18S-F	5′- TGACCGAATGAGCAAGGAAATTACT -3′
18S-R	5′-TACTCAGCTTTGGCAATCCACATC -3′
MdTDC1-F	5′- TCACGCTGTGGTTGGAGGT -3′
MdTDC1-R	5′- CTGCATGCTCCTGAACCAAC -3′
MdTDC3-F	5′- CTGTGTTGTCAGGAAAGTACGTATTAC -3′
MdTDC3-R	5′- AAAGAGGTGCAAGGAGGGC -3′
MdT5H4-F	5′- TCGGTGACATGTTTGCTGC -3′
MdT5H4-R	5′- GGAAACCTTGGTCTGGCG -3′
MdAANAT2-F	5′- GAATCACCGTCCACGCTCC -3′
MdAANAT2-R	5′- GAAATGCTTCCGATGTCCC -3′
MdAANAT5-F	5′- CTGGGCGACGATAGTGGAA -3′
MdAANAT5-R	5′- AATGGCTGCTGTCAGTAGTGCT -3′
MdASMT3-F	5′- AGGAAATACCTCCAGCCGAT -3′
MdASMT3-R	5′- CCTCATTTTTGCTAAGAGATATTGC -3′
MdASMT5-F	5′- TCACCAGCAAAGAGCGTAGC -3′
MdASMT5-R	5′- TCAGAGGTAAACTTCAATAAGAGACC -3′
MdT5H1-F	5′- AGGCATATCCGTAAGATTTGTATACT -3′
MdT5H1-R	5′- TCACCGACCAAGATAATAGCCT -3′
MdAANAT1-F	5′- CCGTCAAGCATCGGATAAGT -3′
MdAANAT1-R	5′- GGAGCGTGGAGGGTGGT -3′
MdAANAT3-F	5′- CGCTCCCTAACTACCAACCA -3′
MdAANAT3-R	5′- ACAAATCCCTTTCCCTACCAG -3′
MdAANAT4-F	5′- GGGAAATGGGAATCGGC -3′
MdAANAT4-R	5′- CACACCCTTTGAAGAATGACCT -3′
MdASMT1-F	5′- AGAGGAGCGAGAAAGACTGGA -3′
MdASMT1-R	5′- CTAAAGAAAAACTTCAATGAGGGAT -3′
MdASMT7-F	5′- GTGCAGCTCAACTGGGCATC -3′
MdASMT7-R	5′- TCCTCCACATCGTCACAATCAT -3′

(DQ341382), MdTDC1 (HF29482), MdTDC3 (MF443139), MdT5H1 (MF443138), MdT5H4 (MF443137), MdAANAT1 (KJ156533), MdAANAT2 (MF443136), MdAANAT3(KJ156532), MdAANAT4 (XM_029105996), MdAANAT5 (KJ156534), MdASMT1 (MF135479), MdASMT3 (KJ156528), MdASMT5 (KT633934), MdASMT7 (KJ156529).

3. Results

3.1. Melatonin affects anthocyanin accumulation but not the growth of crabapple

To investigate the effect of melatonin on crabapple plant development and anthocyanin accumulation, plantlets of the ever-red crabapple cultivar ‘Royalty’ were grown on MS media containing various concentration of melatonin.

The fresh weight increment, shoot height and shoot width of ‘Royalty’ and the cultivar used in later experiments, ‘Radiant’, showed slightly variations following different melatonin treatments after 2 weeks of treatment (Tables 2 and 3), and we concluded that exogenous melatonin application did not affect the growth of crabapple plants.

Treatments with increasing concentrations of melatonin induced a leaf color shift from partial red to purple red in ‘Royalty’ (Fig. 1A). To confirm that the pigmentation was a result of anthocyanin accumulation, we conducted HPLC analysis, which showed that the anthocyanin contents gradually increased with the increased melatonin concentrations, from 50 to 500 μg/L, before decreasing at the highest (1000 μg/L) melatonin concentration. The same trend was observed for the flavonoids compound phlorizin in ‘Royalty’. The levels of procyanidin B2 and (−)-epicatechin were highest following the 50 μg/L and 200 μg/L treatments, respectively, and gradually decreased with increasing melatonin concentration (Fig. 1B).

3.2. The effect of melatonin on the expression levels of anthocyanin-related genes

To investigate the mechanism underlying the melatonin induced anthocyanin accumulation in crabapple leaves, we assessed how changing the concentration of melatonin in the medium affected the transcription of flavonoid biosynthetic and regulatory genes.

Consistent with the variation in flavonoid accumulation, the transcript levels of flavonoid biosynthetic genes (*McCHS, McF3H, McF3′H, McDFR, McANS, McUFGT, McANR1, McANR2, McLAR1* and *McFLS*) progressively increased with 200 μg/L or 500 μg/L melatonin concentrations, expression levels decreased in response to the 500 μg/L or 1000 μg/L melatonin treatments (Fig. 1D), which matched the pattern of anthocyanin accumulation.

qRT-PCR analysis also showed that the expression levels of flavonoid related transcription factors (McMYB4, McMYB10, McbHLH3 and McbHLH33) were significantly induced by melatonin supplementation, and were approximately 20- to 100-fold higher in treated plants than that in control plants. In addition, McMYB10, McbHLH3 and

Table 2
Effect of Melatonin at Different Concentrations on crabapple cultivar ‘Royalty’.

	Plant weight (g)		Plant height (cm)		Plant width(cm)	
	Before	After	Before	After	Before	After
CK	0.12 ± 0.09	0.14 ± 0.02	2.99 ± 0.07	3.08 ± 0.12	2.41 ± 0.10	2.43 ± 0.8
50μg/L	0.11 ± 0.02	0.13 ± 0.02	2.91 ± 0.11	3.05 ± 0.09	2.25 ± 0.06	2.32 ± 0.11
100μg/L	0.12 ± 0.06	0.13 ± 0.11	2.73 ± 0.10	2.85 ± 0.11	2.15 ± 0.11	2.25 ± 0.13
200μg/L	0.11 ± 0.11	0.12 ± 0.02	2.86 ± 0.09	2.94 ± 0.05	2.16 ± 0.12	2.28 ± 0.4
500μg/L	0.12 ± 0.10	0.13 ± 0.02	2.85 ± 0.12	3.01 ± 0.08	2.15 ± 0.08	2.35 ± 0.6
1000μg/L	0.11 ± 0.08	0.13 ± 0.06	2.86 ± 0.009	3.15 ± 0.11	2.43 ± 0.12	2.51 ± 0.6

Data are expressed as mean ± S.D. Data were analyzed using independent t-tests at a significance level of $P < 0.05$ (*).

Table 3
Effect of Melatonin at Different Concentrations on crabapple cultivar 'Radiant'.

	Plant weight (g)		Plant height (cm)		Plant width(cm)	
	Before	After	Before	After	Before	After
CK	0.10 ± 0.02	0.12 ± 0.03	3.29 ± 0.04	3.39 ± 0.10	2.01 ± 0.10	2.13 ± 0.09
50μg/L	0.09 ± 0.01	0.11 ± 0.05*	3.22 ± 0.09	3.35 ± 0.03	1.65 ± 0.06	1.96 ± 0.10
100μg/L	0.10 ± 0.04	0.13 ± 0.03	2.84 ± 0.07	3.10 ± 0.08*	2.05 ± 0.11	2.25 ± 0.11
200μg/L	0.10 ± 0.09	0.12 ± 0.03*	2.96 ± 0.10	2.99 ± 0.07	1.66 ± 0.12	1.87 ± 0.06
500μg/L	0.09 ± 0.07	0.10 ± 0.04	3.12 ± 0.4	3.21 ± 0.04	1.74 ± 0.08	1.89 ± 0.08
1000μg/L	0.10 ± 0.05	0.11 ± 0.02	2.89 ± 0.07	3.05 ± 0.10	2.02 ± 0.12	2.10 ± 0.11

Data are expressed as mean ± S.D. Data were analyzed using independent t-tests at a significance level of $P < 0.05$ (*).

McbHLH33 transcript levels correlated with anthocyanin concentrations and transcripts of anthocyanin biosynthesis genes (*McCHS, McF3H, McF3'H, McDFR, McANS* and *McUFGT*). McMYB12 transcript levels correlated with PA concentrations and the transcript abundance of PA biosynthetic genes (McANR1, McANR2 and McLAR1), while McMYB4 transcript levels correlated with flavonol concentrations and the expression of a flavonol biosynthetic gene (*McFLS*) (Fig. 1C).

3.3. Melatonin alters flavonoid accumulation in a spring-red crabapple cultivar

To investigate whether melatonin promotes anthocyanin accumulation in other crabapple cultivars, we repeated the melatonin treatments in the spring-red crabapple cultivar 'Radiant'. After 2 weeks, the leaves showed an obvious red color accumulation in 'Radiant' (Fig. 2A), and HPLC analysis revealed that the highest anthocyanin content was in the 200 μg/L melatonin treated samples grown in the light (Fig. 2B). The accumulation trend observed for phlorizin in 'Royalty' was also observed in 'Radiant', while the abundance of procyanidin B2 and (−)-epicatechin showed no obvious variation. qRT-PCR analysis showed that most anthocyanin biosynthetic genes were activated by the melatonin treatment and that the highest expression levels were observed following 200 μg/L or 500 μg/L treatments. However, while the transcription of PA biosynthetic genes increased after melatonin treatment, the PA content showed no obvious variation. Finally, the expression levels of flavonoid related transcription factors (McMYB4, McMYB10, McMYB12, McbHLH3 and McbHLH33) were significantly higher after supplementation with melatonin and had similar accumulation trends as the anthocyanin content and the transcription of anthocyanin biosynthetic genes.

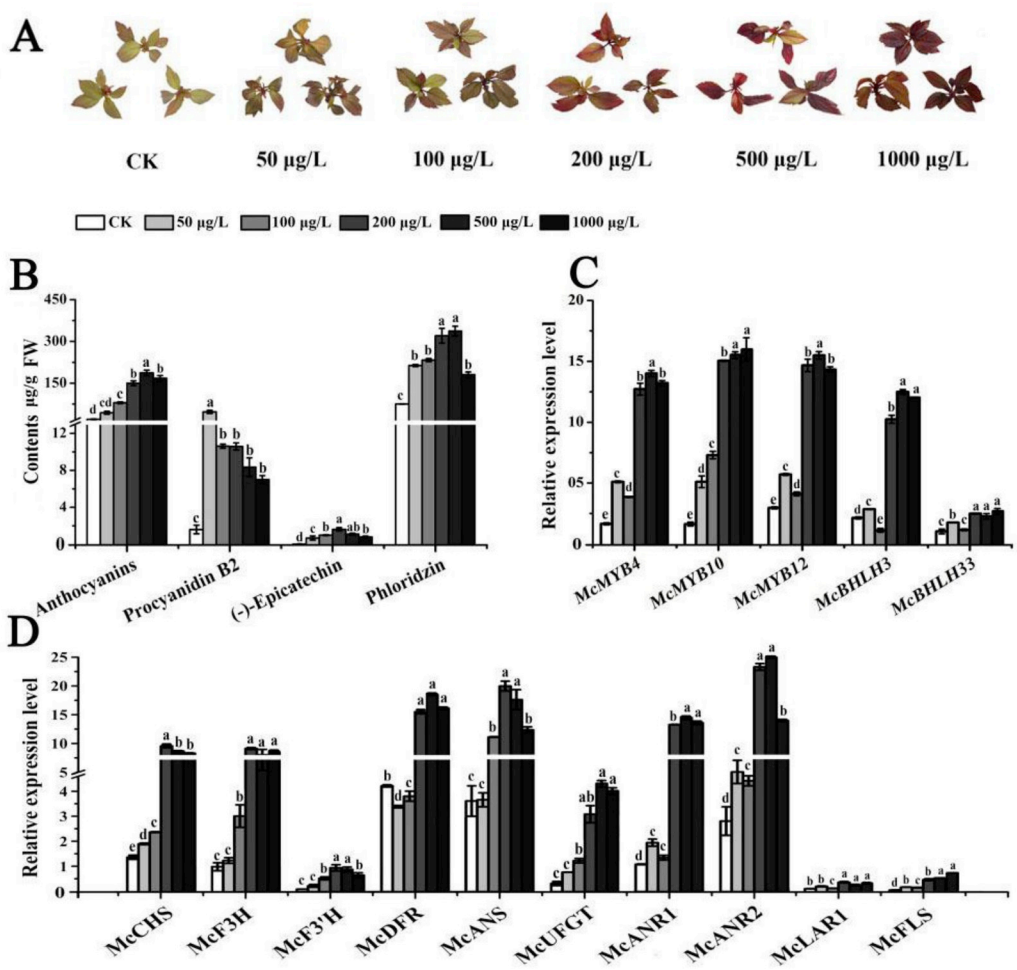

Fig. 1. Treatments with different concentrations of melatonin can alter anthocyanin accumulation in 'Royalty' foliage. (A) Exogenous melatonin induces red coloration of 'Royalty' foliage. (B) High pressure liquid chromatography (HPLC) analysis showing the variation in flavonoid content in crabapple leaves following different melatonin treatments. (C) The expression levels of regulatory flavonoid biosynthetic genes. (D) The expression levels of flavonoid biosynthetic genes (*McCHS*, *McF3H*, *McF3'H*, *McDFR*, *McANS*, *McUFGT*, *McANR1*, *McANR2*, *McLAR1*, *McFLS*). Error bars indicate the standard error of the mean ± SE of three replicate measurements. Different letters above the bars indicate significantly different values ($P < 0.05$) calculated using one-way analysis of variance (ANOVA) followed by a Tukey's multiple range test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

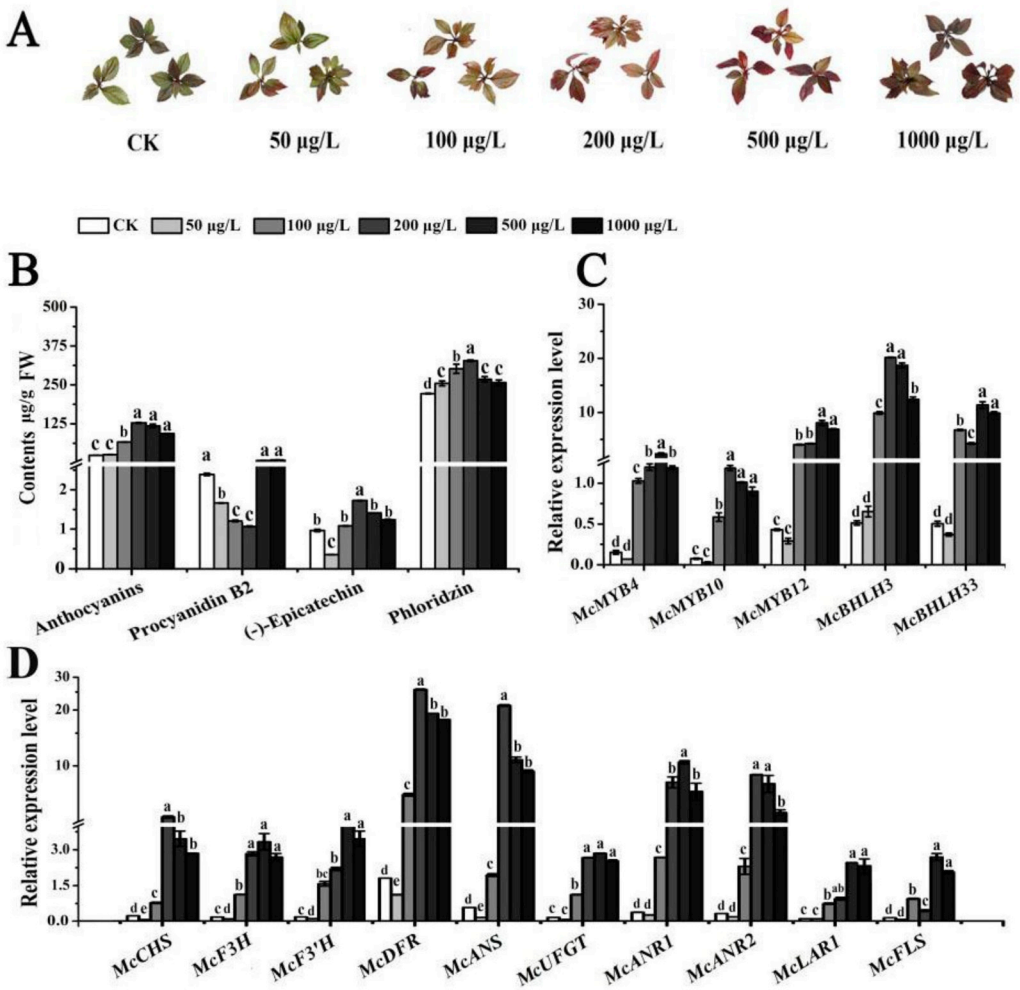

Fig. 2. Treatments with different concentrations of melatonin can alter anthocyanin accumulation in 'Radiant' foliage. (A) Exogenous melatonin induces red coloration in 'Radiant' foliage. (B) High pressure liquid chromatography (HPLC) analysis showing the flavonoid content of crabapple leaves following different melatonin treatments. (C) The expression levels of regulatory flavonoid biosynthetic genes. (D) The expression levels of flavonoid biosynthetic genes (*McCHS*, *McF3H*, *McF3'H*, *McDFR*, *McANS*, *McUFGT*, *McANR1*, *McANR2*, *McLAR1*, *McFLS*). Error bars indicate the standard error of the mean ± SE of three replicate measurements. Different letters above the bars indicate significantly different values ($P < 0.05$) calculated using one-way analysis of variance (ANOVA) followed by a Tukey's multiple range test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Melatonin promoted anthocyanin accumulation is not dependent on light

Light induces anthocyanin accumulation in many plant species, including *A. thaliana* (Rowan et al., 2010), tobacco (*Nicotiana tabacum*) and apple (Takos et al., 2006; Zhou et al., 2008). To understand the effect of melatonin on crabapple leaf coloration and to determine whether it is dependent on light, we placed plantlets of 'Royalty' and 'Radiant' supplied with various concentrations of melatonin in the dark. After 2 weeks, anthocyanin accumulation was significantly higher and the plants displayed a bright red color in the leaf margin of the purple leaf phenotype of 'Royalty', and green in the leaf margin of the red leaf phenotype of 'Radiant' (Fig. 3A and Fig. 4A). HPLC analysis showed that the anthocyanin content was lower than under light conditions, and indicated that the phenotypic variations were due to the significant accumulation of anthocyanins in the two cultivars. The highest anthocyanin levels were observed in 500 μg/L melatonin treated 'Royalty' and 200 μg/L melatonin treated 'Radiant' leaves. We also observed that the higher melatonin concentration promoted the accumulation of other flavonoid compounds (such as PAs) in these two cultivars (Figs. 3B and 4B).

Transcript analysis using qRT-PCR indicated that the expression levels of most of the regulatory genes that affect flavonoid biosynthesis and structural genes in the anthocyanin pathway were correlated with anthocyanin accumulation. These genes were significantly up-regulated in the 100 μg/L melatonin treated plants, and repressed by higher exogenous melatonin (> 200 μg/L) concentrations.

3.5. The expression of melatonin biosynthetic genes during melatonin treatment

The expression of two *McTDC* genes (*McTDC1* and *McTDC3*), two *McT5H* genes (*McT5H1* and *McT5H4*), five *McAANAT* genes (*McAANAT1, McAANAT2, McAANAT3, McAANAT4*, and *McAANAT5*), and four *McASMT* genes (*McASMT1*, *McASMT3, McASMT5,* and *McASMT7*) was detected in melatonin treated crabapple plantlets grown under either light or dark conditions, and showed similar pattern to that of flavonoid accumulation: the expression of melatonin biosynthetic genes is also activated by the exogenous application of melatonin (Figs. 5 and 6). Correlation analysis also showed that the expression of melatonin biosynthetic genes was closely correlated with anthocyanin abundance in crabapple leaves; notably *McASMT3* and *McAANAT4* in 'Royalty' and in 'Radiant' light grown plants. The expression of melatonin biosynthetic genes showed a different pattern in 'Radiant' grown under dark conditions, where expression of *McTDC3* and *McT5H1* correlated with anthocyanin content. We therefore concluded that these genes may play a role in anthocyanin accumulation in 'Radiant' grown under these conditions (Table 4).

4. Discussion

In 1995, the first report of melatonin in higher plants was published (Dubbels et al., 2010; Hattori et al., 1995; Tan et al., 2015) and subsequently its function in several plant development processes, and in promoting tolerance to abiotic and biotic stresses, such as cold (Jiang et al., 2016; Shiu and Pang, 1984; Li et al., 2017), drought (Kabiri et al.,

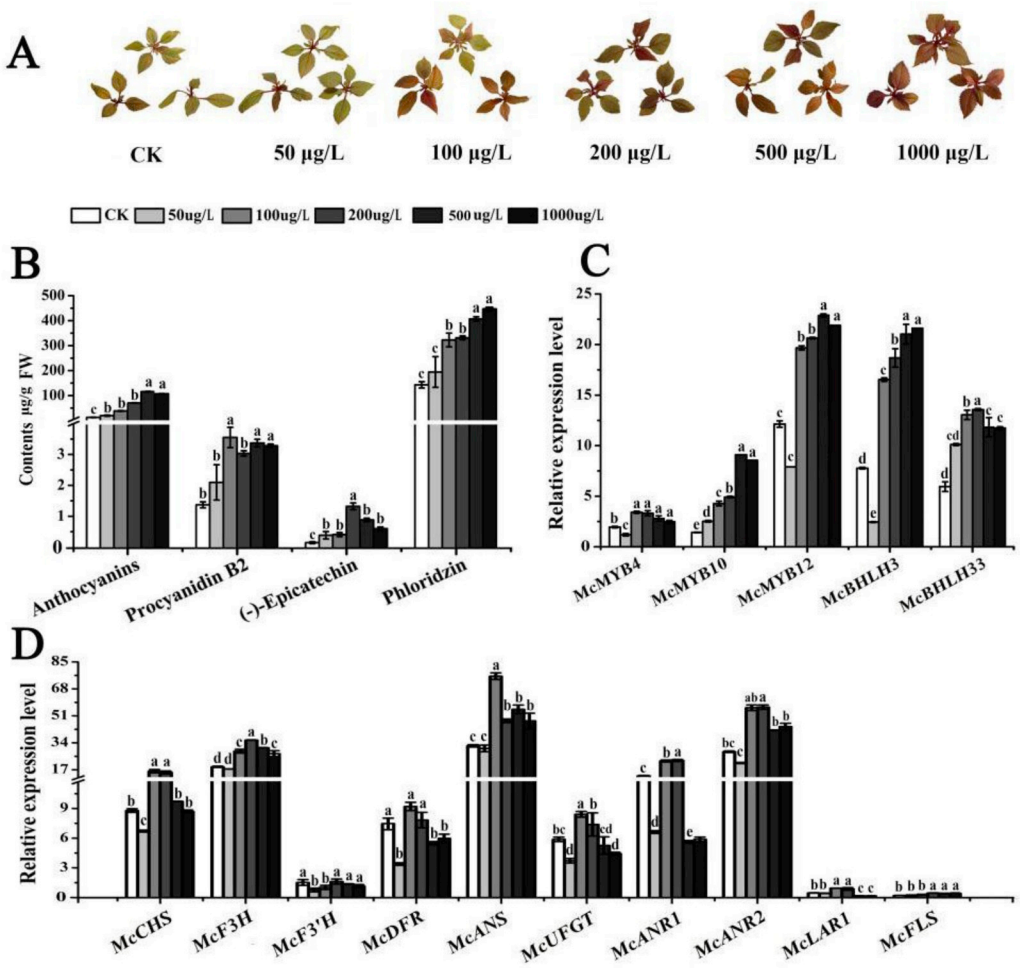

Fig. 3. Exogenous melatonin can alter anthocyanin accumulation in 'Royalty' foliage under dark conditions. (A) Exogenous melatonin induces red coloration in 'Royalty' foliage under dark conditions. (B) High pressure liquid chromatography (HPLC) analysis showing the variation in flavonoids in crabapple leaves following different melatonin treatments. (C) The expression levels of regulatory flavonoid biosynthetic genes. (D) The expression levels of flavonoid biosynthetic genes (*McCHS*, *McF3H*, *McF3'H*, *McDFR*, *McANS*, *McUFGT*, *McANR1*, *McANR2*, *McLAR1*, *McFLS*). Error bars indicate the standard error of the mean ± SE of three replicate measurements. Different letters above the bars indicate significantly different values ($P < 0.05$) calculated using one-way analysis of variance (ANOVA) followed by a Tukey's multiple range test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2018; Cui et al., 2017), salt (Hardeland et al., 2011; Li et al., 2012), heavy metals (Liang et al., 2015; Posmyk et al., 2010), UV and ozone damage (Tan et al., 2007), and diseases (Hernándezruiz and Arnao, 2008a,b; Arnao and Hernández-Ruiz, 2014) has been described. It was also reported that melatonin may regulate the phenylpropanoid pathway to promote stress resistance, a pathway that leads to the synthesis of anthocyanins, which have similar protective roles (Fleta-Soriano et al., 2017). To investigate the potential relationship between melatonin and anthocyanin, we applied exogenous melatonin to crabapple plantlets and measured anthocyanin accumulation, as well as examining the expression of anthocyanin biosynthetic genes in crabapple leaves.

It has been suggested that melatonin may regulate plant growth as application of exogenous melatonin promotes seedling development and root growth in maize (*Zea mays*), wheat (*Triticum aestivum*) and *A. thaliana* (Wang et al., 2013). However, in this current study, exogenous melatonin did not promote crabapple plant growth, since the fresh weight, plant height and plant width was not significantly different after supplementation with different concentrations of melatonin. This may be because crabapple is a woody plant, unlike the above species. However, the exogenous melatonin treatment did promote anthocyanin accumulation in the crabapple leaves. The leaves of the ever-red cultivar 'Royalty' and the spring-red cultivar 'Radiant' also exhibited the purple red leaf phenotype.

qRT-PCR analysis revealed that anthocyanin biosynthetic genes were strongly induced by the exogenous melatonin treatment. Specifically, the expression of *McCHS, McF3H, McF3'H, McDFR, McANS* and *McUFGT* was increased together with the accumulation of anthocyanins, as was the expression of McMYB10, McbHLH3 and McbHLH33. We propose that melatonin may trigger red foliage coloration by activating anthocyanin-related transcription factors, which in turn activate genes in various steps of the anthocyanin biosynthetic pathway.

In oat (*Avena sativa*) coleoptiles, treatment with exogenous melatonin concentrations < 0.01 μM promoted growth, while a 0.1 μM melatonin treatment resulted in maximum growth inhibition (Wang et al., 2013). In maize seedlings, it was shown that exogenous 10 μM melatonin was the optimal concentration for promoting seedling growth, while growth inhibition was observed at higher doses. In wheat, exogenous melatonin promoted root growth at concentrations < 1 μM, whereas concentrations ≥10 μM inhibited root growth (Yin et al., 2013). We showed that 200 μg/L melatonin was the optimal concentration for anthocyanin accumulation in two crabapple cultivars under light conditions, and that the anthocyanin content did not increase with higher doses. These data indicate that exogenous melatonin application regulates anthocyanin biosynthesis in a dose-dependent manner.

Light plays an important role in flavonoid production and has been shown to significantly increase the accumulation of flavonoids and the expression of their biosynthetic genes in grape (*Vitis vinifera*) (Jessica and James, 2006; Jeong et al., 2004). In apple, the expression of MdMYB1 increased within 1 day of exposure of the fruit skin to light and anthocyanin accumulation was initiated in the same time frame (Otteneder et al., 2010). We note that exogenous melatonin promotion of anthocyanin is not dependent on light as the crabapple plantlets also accumulated anthocyanins under dark conditions after treatment with melatonin, even though the contents were lower than under light conditions. The results presented here provide additional evidence that

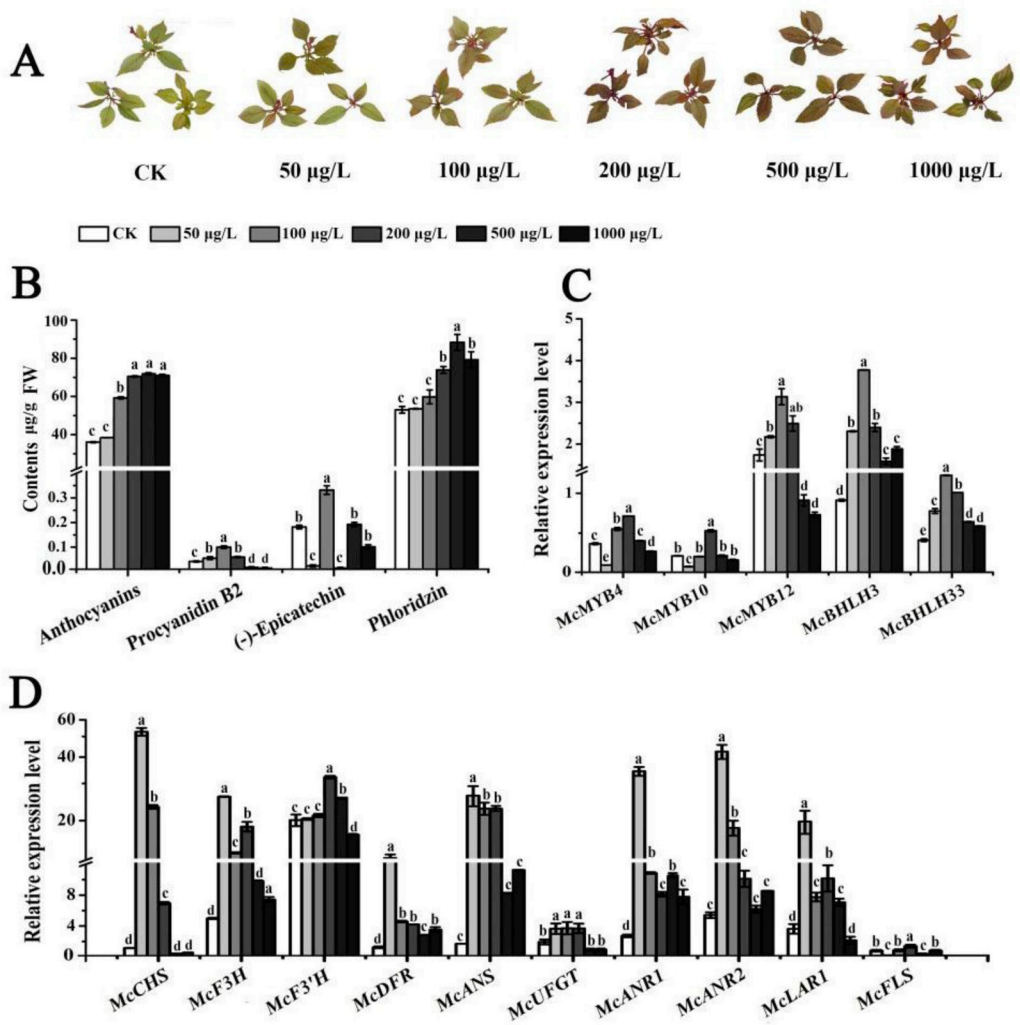

Fig. 4. Exogenous melatonin can alter anthocyanin accumulation in 'Radiant' foliage under dark conditions. (A) Exogenous melatonin induces red colorstion in 'Radiant' foliage under dark conditions. (B) High pressure liquid chromatography (HPLC) analysis showing the variation in flavonoids in crabapple leaves following different melatonin treatments. (C) The expression levels of regulatory flavonoid biosynthetic genes. (D) The expression levels of flavonoid biosynthetic genes (*McCHS*, *McF3H*, *McF3'H*, *McDFR*, *McANS*, *McUFGT*, *McANR1*, *McANR2*, *McLAR1*, *McFLS*). Error bars indicate the standard error of the mean ± SE of three replicate measurements. Different letters above the bars indicate significantly different values ($P < 0.05$) calculated using one-way analysis of variance (ANOVA) followed by a Tukey's multiple range test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

melatonin may directly activate the expression of anthocyanin-related transcription factors.

Anthocyanins are antioxidants and have beneficial effects on plant and human health and there is interest in developing strategies to increase their production in crops for both ornamental and medical uses (Jing et al., 2015). Flavonols and PAs also play important roles in protection against ultraviolet radiation and other environmental stresses (Matus, 2016). In plants, R2R3-MYB TF family has been demonstrated that it may be the main anthocyanins, PAs and flavonol biosynthesis regulators (Espley et al., 2007; Stracke et al., 2001; Wang et al., 2017). In pear (*Pyrus bretschneideri*), PbMYB9 not only acts as activator of the PA biosynthetic pathway by activating the *PbANR* (anthocyanidin reductase) promoter, but also induces the accumulation of anthocyanins and flavonols by binding to the promoter of *PbUFGT1* (UDP-glucose flavonoid 3-*O*-glucosyltransferase) (Zhai et al., 2016). In grapevine, transient expression of VvMYB5a and VvMYB5b in tobacco (*Nicotiana tabacum*) resulted in the activation of several flavonoid pathway genes, and when overexpressed, the biosynthesis of anthocyanins, flavonols, tannins and lignins in reproductive organs was increased (Deluc et al., 2006, 2008). In apples (*Malus sieversii* f. *niedzwetzkyana*), MdMYB22 was found to promote the flavonol accumulation and PAs by directly binding the *MdFLS* and *MdLAR* promoter (Wang et al., 2017). Meanwhile, environmental factors also impact the flavonoid accumulation in many plants. Low temperature can promote anthocyanin and flavonol accumulation in crabapple cultivar 'Royalty' by inducing the expression of several anthocyanin biosynthetic and regulation genes (Tian et al., 2015a). Meanwhile, light treatment can increase the content of anthocyanin and flavonol by activating the SPL TFs in apple fruits. Here, we showed that the content of anthocyanin, flavonols and PAs increased in both cultivars following melatonin treatment, suggesting a promotion of the entire flavonoid biosynthetic pathway (Yang et al., 2019). Given that melatonin is not toxic to animals and humans, its exogenous application may represent an excellent strategy to elevate flavonoid contents in plants. Here, we showed that the content of anthocyanin, flavonols and PAs increased in both cultivars following melatonin treatment, suggesting a promotion of the entire flavonoid biosynthetic pathway. Given that melatonin is not toxic to animals and humans, its exogenous application may represent an excellent strategy to elevate flavonoid contents in plants.

5. Conclusions

In conclusion, we examined the effects of exogenous melatonin on anthocyanin accumulation in crabapple plants. We conclude that melatonin may increase anthocyanin levels by directly activating anthocyanin biosynthesis related transcription factors, which then activate the expression of anthocyanin biosynthetic genes. Melatonin triggered anthocyanin biosynthesis was shown to be light independent and we observed that the application of melatonin to crabapple plants also promoted the accumulation of PAs and flavonols. This study provides insights into the mechanism by which melatonin regulates anthocyanin production in woody plants, and shows that exogenous pretreatment of melatonin may represent a promising cultivation strategy to increase flavonoid contents in plants.

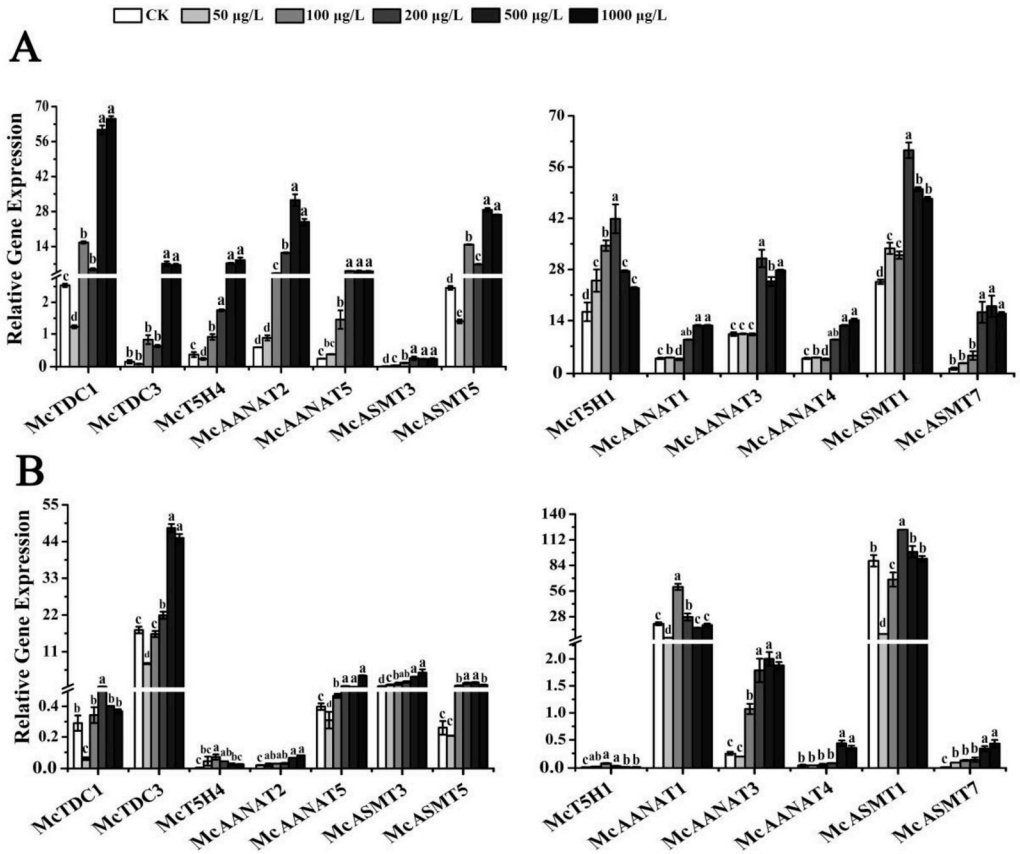

Fig. 5. Expression profiling of melatonin biosynthetic genes following melatonin treatment under light and dark conditions in 'Royalty' leaves. (A) The expression of melatonin biosynthetic genes following melatonin treatment in 'Royalty' under light conditions. (C) The expression of melatonin biosynthetic genes following melatonin treatment in 'Royalty' under dark conditions. Error bars indicate the standard error of the mean ± SE of three replicate measurements. Different letters above the bars indicate significantly different values ($P < 0.05$) calculated using one-way analysis of variance (ANOVA) followed by a Tukey's multiple range test.

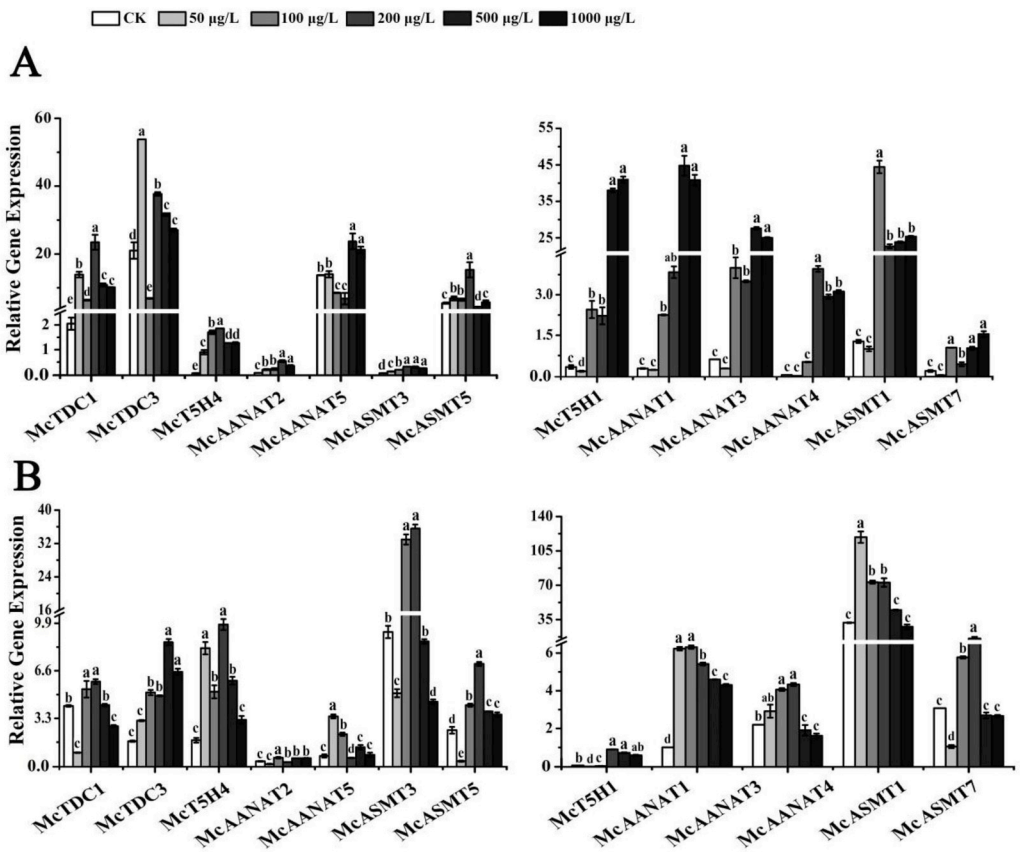

Fig. 6. Expression profiling of melatonin biosynthetic genes following melatonin treatment under light and dark conditions in 'Radiant'. (A) The expression of melatonin biosynthetic genes following melatonin treatment in 'Radiant' under light conditions. (B) The expression of melatonin biosynthetic genes following melatonin treatment in 'Radiant' under dark conditions. Error bars indicate the standard error of the mean ± SE of three replicate measurements. Different letters above the bars indicate significantly different values ($P < 0.05$) calculated using one-way analysis of variance (ANOVA) followed by a Tukey's multiple range test.

Table 4
Correlation analysis between the transcription of melatonin biosynthetic genes and anthocyanin accumulation in crabapple leaves during growth under light and dark conditions.

		McTDC1	McTDC3	McT5H4	McAANAT2	McAANAT5	McASMT3	McASMT5	McT5H1	McAANAT1	McAANAT3	McAANAT4	McASMT1	McASMT7
Light	'Royalty'	0.418	0.125	0.091	0.318	0.500	0.963**	−0.049	0.388	0.934**	0.845*	0.934**	0.890*	0.974**
Anthocyanin	'Radiant'	0.416	0.904*	0.138	0.920**	0.588	0.950**	0.902*	−0.008	0.187	0.944**	0.868*	0.581	0.944**
dark	'Royalty'	0.542	0.564	0.648	0.764	0.955**	0.985**	0.584	0.576	0.686	0.702	0.961**	0.427	0.412
Anthocyanin	'Radiant'	0.236	0.842*	−0.005	0.573	−0.400	0.147	0.125	0.864*	−0.114	0.053	0.111	−0.375	0.295

Data were analyzed using independent t-tests at a significance level of $P < 0.05$ (*).

Author contributions

Conceived and designed the experiments: JT JZ YY. Performed the experiments: LC SW. Analyzed the data: LC JT YY. Contributed reagents/materials/analysis tools: JZ YY TS. Wrote the paper: LC JT YY.

Acknowledgements

We thank the Fruit Tree Key Laboratory at the Beijing University of Agriculture and the Beijing Nursery Engineering Research Center for Fruit Crops for providing experimental resources. We are grateful to all technicians in the BUA Crabapple Germplasm Resource Garden. We also thank PlantScribe (www.plantscribe.com) for editing this manuscript. Financial support was provided by the 'The Project of Construction of Innovative Teams and the Teacher Career Development for Universities and Colleges Under Beijing Municipality' (IDHT20180509), 'The Construction of Beijing Science and Technology Innovation and Service Capacity in Top Subjects' (CEFF-PXM2019_014207_000032), 'Cultivating High Level Teachers in Colleges and Universities in Beijing' (5045243001), 'The National Natural Science Foundation of China' (31772263).

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