DOI: 10.1007/s10535-014-0472-z BIOLOGIA PLANTARUM 59 (1): 99-105, 2015

Brassinosteroid enhances cytokinin-induced anthocyanin biosynthesis in *Arabidopsis* seedlings

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Abstract

To investigate whether brassinosteroids (BR) affects cytokinin (CK)-induced anthocyanin biosynthesis, seedlings of the *Arabidopsis dwarf4* (*dwf4*) mutants including *partially suppressing coi1* (*psc1*) and *dwf4-102*, which are defective in the BR biosynthesis, and the *brassinosteroid-insensitive 1-4* (*bri1-4*) mutant defective in BR signalling were used for the analysis of CK-induced anthocyanin accumulation and the expression of anthocyanin biosynthetic genes and WD-repeat/Myb/bHLH transcription factors. The results show that the CK-induced anthocyanin accumulation was remarkably reduced in *dwf4* and *bri1-4* mutants, but distinctly increased in the wild type (WT) treated with BR. Moreover, the CK-induced expressions of the late anthocyanin biosynthetic genes including *dihydroflavonol reductase*, *leucoanthocyanidin dioxygenase*, and *UDP-glucose: flavonoid-3-O-glucosyl transferase* were significantly reduced in *bri1-4* and *dwf4-102* mutants compared to WT. In addition, the expressions of transcription factors *production of anthocyanin pigment 1* (*PAP1*), *glabra 3* (*GL3*), and *enhancer of glabra 3* (*EGL3*) were induced by CK in WT but not in the *bri1-4* and *dwf4-102* mutants. These results indicate that BR enhanced the CK-induced anthocyanin biosynthesis by up-regulating the late anthocyanin biosynthetic genes and this regulation might be mediated by the transcription factors *PAP1*, *GL3*, and *EGL3*.

Additional key words: anthocyanin biosynthetic genes, *BRI1*, mutants, *PSC1/DWF4*, WD-repeat/Myb/bHLH transcription factors.

Introduction

Anthocyanins, the ubiquitous group of water-soluble plant metabolites of the flavonoid family, are responsible for the most widespread pigment classes in higher plants (Springob *et al*. 2003). Anthocyanins also play an important role in plant defense against abiotic stresses, including UV radiation, drought, and low temperature, and in plant-microbe interactions (Winkel-Shirley 2002, Gould 2004, Hatier and Gould 2008, Gould *et al*. 2010, Cooney *et al*. 2012, Hatier *et al*. 2013).

Anthocyanin biosynthesis, as branch of flavonoid pathway, has been extensively studied (Holton and Cornish 1995, Grotewold 2006, Chen *et al*. 2012). Genes encoding enzymes required for the anthocyanin biosynthetic pathway are divided into two groups, the early anthocyanin biosynthetic genes, such as *chalcone synthase* (*CHS*) and *chalcone isomerase* (*CHI*), and the late anthocyanin biosynthetic genes including *dihydroflavonol reductase* (*DFR*), *leucoanthocyandin*

Submitted 6 November 2013, *last revision* 26 June 2014, *accepted* 8 July 2014.

Abbreviations: BR - brassinosteroids; *CHI* - *chalcone isomerase*; *CHS* - *chalcone synthase*; CK - cytokinin; *DFR* - *dihydroflavonol reductase*; *EGL3* - *enhancer of glabra 3*; *GL3* - *glabra 3*; JA - jasmonate; *LDOX* - *leucoanthocyanidin dioxygenase*; *PAP1/2* - *production of anthocyanin pigment 1/2*; *TTG1* - *transparent testa glabra 1*; *UF3GT* - *UDP-glucose*: *flavonoid-3-O-glucosyl transferase*.

Acknowledgements: This work was supported by grants from the National Science Foundation of China (30770195), the Hunan Provincial Natural Science Foundation of China (12JJ2021), the Scientific Research Fund of Hunan Provincial Education Department (12C0166), the Hunan Provincial Graduate Student's Innovative Research (CX2013B301), and the Program for Innovative Research Team in University (IRT1239). The authors L.B. YUAN, Z.H. PENG, and T.T. ZHI contributed equally to this paper.

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dioxygenase (*LDOX*), and *UDP-glucose: flavonoid-3-O-glucosyl transferase* (*UF3GT*) (Holton and Cornish 1995, Pelletier *et al*. 1997, Grotewold 2006). WD-repeat/ Myb/bHLH transcription complexes including transcription factors *production of anthocyanin pigment1* (*PAP1*), *PAP2*, *glabra 3* (*GL3*), *enhancer of glabra 3* (*EGL3*), and *transparent testa glabra 1* (*TTG1*) mainly regulate the expression of the late anthocyanin biosynthetic genes (Gonzalez *et al*. 2008).

The anthocyanin biosynthesis is stimulated by environmental stresses, such as wounding, UV irradiation, drought, nutrient deficiency, and pathogen attack (Winkel-Shirley 2002). Sugars induce the anthocyanin biosynthesis (Solfanelli *et al*. 2006). Plant hormones, including cytokinins (CK) and jasmonates (JA), are also involved in the regulation of the anthocyanin biosynthesis (Deikman and Hammer 1995, Shan *et al*. 2009, Qi *et al*. 2011). CK significantly induce the expression of both the early and late anthocyanin biosynthetic genes (Deikman and Hammer 1995), whereas JA induce only the expression of the late anthocyanin biosynthetic genes (Shan *et al*. 2009).

Brassinosteroids (BR) are steroidal hormones that regulate a wide range of physiological processes during plant growth and development (Yokota 1997, Sasse 2003), and play a crucial role in response to various stresses (Krishna 2003, Bajguz and Hayat 2009). In addition, BR interact with other hormones in regulating many developmental processes and stress responses (Gendron *et al*. 2008, Ren *et al*. 2009, Huang *et al*. 2010, Choudhary *et al*. 2012). Previously, we found that BR enhances JA-induced anthocyanin accumulation (Peng *et al*. 2011). The aim of the present study was to investigate whether BR affect the CK-induced anthocyanin biosynthesis using physiological and genetic approaches.

Materials and methods

Arabidopsis thaliana L. ecotypes Columbia (Col-0) and Wassilewskija (Ws-2) were used as the wild types (WTs) in this study. The *dwarf4-102* (*dwf4-102*) (Nakamoto *et al*. 2006, SALK_020761) and *brassinosteroid-insensitive 1-4* (*bri1-4*) (Noguchi *et al*. 1999) mutants, which are in the Col-0 and Ws-2 background, respectively, were ordered from the Arabidopsis Biological Resource Center. The *partially suppressing coi1* (*psc1*) mutant was isolated by Ren *et al*. (2009).

Seeds were surface-sterilized with 20 % (v/v) chlorine bleach containing 0.1 % (v/v) *Triton X-100* for 10 min, washed five times with sterile water, plated on a half-strength Murashige and Skoog (MS) medium (*Sigma*, Saint Louis, USA) supplemented with 30 mM sucrose (pH 5.8), chilled at 4 °C for 3 d, and then transferred to a growth chamber with a 16-h photoperiod, an irradiance of 150 μmol m^{-2} s^{-1}, a temperature of 22 - 24 °C, and a relative humidity of 65 %.

For CK treatment, 7-d-old seedlings grown on the 1/2 MS medium were transferred onto a 1/2 MS medium containing 90 mM sucrose with or without 0.44 μM 6-benzylaminopurine (BA, *Sigma*) for an additional 4 d.

For treatment with CK in the presence of BR, 7-d-old seedlings were transferred onto a 1/2 MS medium containing 90 mM sucrose with or without 0.1 μM 24-epibrassinolide (epi-BL; *Sigma*) and with or without 0.44 μM BA for an additional 4-d growth.

The measurement of anthocyanin content was performed as described by Deikman and Hammer (1995). Preweighed seedlings were placed into 1 cm^3 of an extraction buffer consisting of 18 % (v/v) 1-propanol and 1 % (v/v) HCl, boiled for 3 min and then incubated in darkness overnight at room temperature. The absorbances were measured at 535 and 650 nm using a spectro-photometer *UV-1700* (*Shimadzu*, Kyoto, Japan). There were 12 samples per each treatment.

Total RNA was isolated using a *TRIzol* reagent (*Invitrogen*, Carlsbad, CA, USA). After incubation with

Table 1. Primers of genes tested by qPCR.

Gene	Forward primer	Reverse primer
CHS	5'-CGCATCACCAACAGTGAACAC-3'	5'- TCCTCCGTCAGATGCATGTG-3'
CHI	5'- CCGGTTCATCGATCCTCTTC-3'	5'-ATCCCGGTTTCAGGGATACTATC-3'
DFR	5'-CCTTATCACCGCGCTCTCT-3'	5'-TGTCCTTGTCTTATGATCGAGTAATGC-3'
LDOX	5'-TCAATTTGGCCTAAGACACCAAGT-3'	5'-TCGCTAGCAAACGAAGACACTT-3'
UF3GT	5'-CAACTGGTTTTCCGTTTCTGGTT-3'	5'-GCTTCCTCGACGGTTGATACAC-3'
PAP1	5'-GACATTACGCCCATTCCTACAAC-3'	5'-TCGAGGTCGAGGCTTATAAACATT-3'
PAP2	5'-GAGGAAAGGTGCATGGACTG-3'	5'-ATCGCATCAGCTTCTTGGT-3'
GL3	5'-TCGGTTCGTTTGGTAATGAGG-3'	5'-GCTTGCAATTGACGGTTAAGC-3'
EGL3	5'-GGAAGACGATTCAAGCAGCA-3'	5'-GGATTCAGCGAGGGAGAGAG-3'
TTG1	5'-GTCATGAACCTCTTTATCAT-3'	5'-ATGGATAATTCAGCTCCAGA-3'
ACTIN2	5'-AGCACTTGCACCAAGCAGCATG-3'	5'-ACGATTCCTGGACCTGCCTCATC-3'

DNase (*Thermo Fisher Scientific*, Waltham, USA) at 37 °C for 30 min and then at 65 °C for 10 min to remove genomic DNA, the RNA sample was quantified with a spectrophotometer *ND-1000* (*NanoDrop*, USA). Reverse transcription was performed using a *ReverTraAce* kit (*Toyobo*, Osaka, Japan). Real-time quantitative PCR (RT-qPCR) was carried out using a *SYBR* qPCR mix (*Toyobo*) with the *ABI 7300* sequence detection system (*Applied Biosystems*, Foster City, CA, USA) following the manufacturer's instructions. Primers of genes tested by qPCR are listed in Table 1, and *ACTIN2* was used as internal control. The gene expression for each sample was calculated on three analytical replicates, and the relative expression was quantified using the $2^{-\Delta\Delta Ct}$ method.

All the experiments were repeated at least three times. Data in the tables are expressed as means ± SE of three biological replicates for qPCR and six biological replicates for the anthocyanin measurement. The significance of differences between data sets was evaluated using the two-tailed Student's *t*-test.

Results

To investigate whether BR affected the CK-induced anthocyanin accumulation, *Arabidopsis* WT Col-0 and BR-deficient mutant *psc1* (Ren *et al*. 2009), a leaky mutation of *DWF4* gene encoding a key enzyme required for BR biosynthesis (Choe *et al*. 1998), were treated with BA following an approach used by Shan *et al*. (2009). Upon the BA treatment, pigmentation appeared in both WT and *psc1*, but lighter in the *psc1* mutant (Fig. 1*A*). The anthocyanin content was significantly reduced in the *psc1* mutant compared to WT in the presence of BA (Table 2). We also determined the anthocyanin content in the seedlings of *dwf4-102*, a null mutant of *DWF4* (Nakamoto *et al*. 2006). The content of anthocyanin in *dwf4-102* mutant was about one-third of that in WT in response to BA (Table 2). We further tested whether an application of exogenous BR influenced the CK-induced anthocyanin accumulation. The WT seedlings were treated with epi-BL and/or BA. As we expected, the seedlings treated with epi-BL showed more obvious pigmentation in the presence of BA (Fig. 1*B*) and their anthocyanin content increased (Table 3).

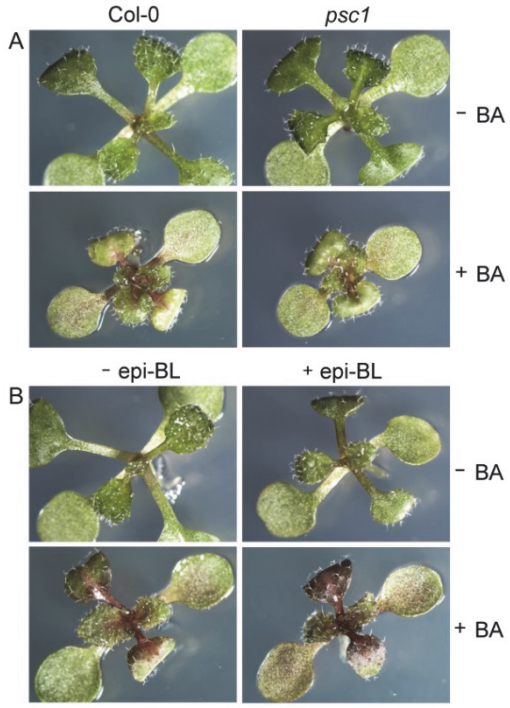

Fig. 1. The effect of BR on cytokinin-induced anthocyanin accumulation. *A* - The phenotype of 7-d-old seedlings of WT (Col-0) and *psc1* mutant treated (+) or untreated (-) with BA for an additional 4 d; *B* - the phenotype of 7-d-old WT seedlings treated (+) or untreated (-) with BA in the absence (-) or presence (+) of epi-BL for an additional 4 d.

Next, we determined whether BR signalling influenced the CK-induced anthocyanin accumulation. BR signalling mutant *bri1-4*, a null allele of *BRI1* that encodes a BR receptor (Noguchi *et al*. 1999), was used for the analysis of anthocyanin accumulation. When seedlings were treated with BA, the anthocyanin content in *bri1-4* was significantly reduced and was less than a half of that in WT Ws-2 (Table 4).

Table 2. Anthocyanin content [$(A_{535} - A_{650})\ g^{-1}$(f.m.)] in seedlings of *psc1*, *dwf4-102*, and WT (Col-0) treated or untreated with BA. Means ± SE, $n = 6$, means with different letters within the same column are significantly different ($P < 0.05$, *t*-test).

Genotypes	Without BA	With BA
Col-0	3.94 ± 0.21a	21.88 ± 0.78a
psc1	2.33 ± 0.30b	14.69 ± 0.43b
dwf4-102	2.13 ± 0.27b	7.00 ± 0.74c

Table 3. Anthocyanin content [$(A_{535} - A_{650})\ g^{-1}$(f.m.)] in WT (Col-0) seedlings treated or untreated with BA in the absence (-) or presence (+) of epi-BL. Means ± SE, $n = 6$, means with different letters within the same column are significantly different ($P < 0.05$, *t*-test).

Epi-BL	Without BA	With BA
- epi-BL	3.96 ± 0.24a	22.13 ± 0.69a
+epi-BL	7.05 ± 0.77b	27.45 ± 0.81b

Table 4. Anthocyanin content [$(A_{535} - A_{650})\ g^{-1}$(f.m.)] in *bri1-4* and WT (Ws-2) seedlings treated or untreated with BA. Means ± SE, $n = 6$, means with different letters within the same column are significantly different ($P < 0.05$, *t*-test).

Genotypes	Without BA	With BA
Ws-2	1.87 ± 0.13a	7.84 ± 0.65a
bri1-4	1.82 ± 0.22a	3.07 ± 0.30b

Table 5. The relative expression of anthocyanin biosynthesis genes including *CHS*, *CHI*, *DFR*, *LDOX*, and *UF3GT* in BR mutants, *bri1-4* and *dwf4-102*, and their WTs, Ws-2 and Col-0, respectively, treated or untreated with BA. The expressions of WTs without BA were considered as 1. Means ± SE, $n = 3$, means with different letters within the same line show significant differences ($P < 0.05$, *t*-test).

Genes	Without BA	With BA	With BA
	bri1-4	Ws-2	*bri1-4*
CHS	1.65 ± 0.23a	3.77 ± 0.46b	5.61 ± 1.13b
CHI	2.70 ± 0.50b	3.93 ± 0.42b	5.67 ± 1.07b
DFR	0.74 ± 0.09a	7.29 ± 0.92c	3.20 ± 0.32b
LDOX	0.82 ± 0.15a	6.47 ± 0.71c	3.84 ± 0.38b
UF3GT	0.68 ± 0.19a	10.12 ± 0.99c	3.35 ± 0.54b
	dwf4-102	Col-0	*dwf4-102*
CHS	1.72 ± 0.23b	3.06 ± 0.45b	3.37 ± 0.89b
CHI	2.19 ± 0.32b	2.82 ± 0.57b	4.05 ± 0.96b
DFR	0.66 ± 0.05b	6.77 ± 0.54d	2.96 ± 0.56c
LDOX	0.69 ± 0.11b	5.78 ± 0.69d	2.74 ± 0.47c
UF3GT	0.46 ± 0.06b	14.25 ± 1.49d	1.96 ± 0.16c

To investigate the molecular mechanism for the effect of BR on the CK-induced anthocyanin accumulation, the expressions of anthocyanin biosynthetic genes namely *CHS*, *CHI*, *DFR*, *LDOX*, and *UF3GT* in *bri1-4* and *dwf4-102* were analyzed by qPCR. Upon the BA treatment, the expressions of *CHS* and *CHI* in both *bri1-4* and *dwf4-102* were comparable to those in their WTs (Table 5), however, the expressions of *DFR*, *LDOX*, and *UF3GT*, which are classified as the late anthocyanin biosynthetic genes, were significantly reduced in both *bri1-4* and *dwf4-102* compared to their WTs (Table 5).

Table 6. The relative expression of WD-repeat/Myb/bHLH complexes transcription factors including *PAP1*, *PAP2*, *GL3*, *EGL3*, and *TTG1* in BR mutants, *bri1-4* and *dwf4-102*, and their WTs, Ws-2 and Col-0, respectively, treated or untreated with BA. The expressions of WTs without BA were considered as 1. Means ± SE, $n = 3$, means with different letters within the same line show significant differences ($P < 0.05$, *t*-test).

Genes	Without BA	With BA	With BA
	bri1-4	Ws-2	*bri1-4*
PAP1	0.81 ± 0.26a	3.08 ± 0.51b	2.03 ± 0.49ab
PAP2	1.23 ± 0.13a	1.51 ± 0.18a	3.99 ± 0.52b
GL3	0.95 ± 0.29a	2.76 ± 0.65b	1.34 ± 0.38ab
EGL3	0.94 ± 0.08a	2.65 ± 0.42b	1.24 ± 0.19ab
TTG1	1.06 ± 0.14a	1.18 ± 0.09a	2.08 ± 0.52a
	dwf4-102	Col-0	*dwf4-102*
PAP1	1.02 ± 0.23a	4.35 ± 0.59b	2.52 ± 0.87ab
PAP2	1.39 ± 0.52a	1.33 ± 0.18a	2.10 ± 0.89a
GL3	0.76 ± 0.28a	4.04 ± 0.68b	2.13 ± 0.48ab
EGL3	0.69 ± 0.27a	3.65 ± 0.74b	1.27 ± 0.31ab
TTG1	1.33 ± 0.31a	1.40 ± 0.29a	1.41 ± 0.33a

Since WD-repeat/Myb/bHLH transcription complexes including transcription factors *PAP1*, *PAP2*, *GL3*, *EGL3*, and *TTG1* mediates anthocyanin biosynthesis mainly by up-regulating the expression of late anthocyanin biosynthetic genes (Gonzalez *et al*. 2008), we further analyzed the expression of these transcription factors in *bri1-4* and *dwf4-102*. The expressions of *PAP1*, *GL3*, and *EGL3* were significantly increased by BA in WTs (Ws-2 and Col-0) but not in *bri1-4* and *dwf4-102* mutants (Table 6). The expressions of *PAP2* and *TTG1* were not remarkably induced by BA in WTs as well as in *bri1-4* and *dwf4-102* except that an increase of the *PAP2* expression in *bri1-4* mutant by the BA treatment was significant (Table 6).

Discussion

Anthocyanin biosynthesis is regulated by a series of endogenous developmental and environmental signals, such as sugar, irradiance, and plant hormones (Deikman and Hammer 1995, Solfanelli *et al*. 2006, Aza-González *et al*. 2013, Shin *et al*. 2013). The signals involved in the anthocyanin biosynthesis do not work independently, and crosstalk and interactions between sucrose, irradiance, and plant hormones occur in regulating the anthocyanin biosynthesis (Guo *et al*. 2005, Loreti *et al*. 2008, Das *et al*. 2012a). For example, the sucrose-induced anthocyanin accumulation is enhanced by JA, CK, and abscisic acid (Loreti *et al*. 2008, Das *et al*. 2012b) but repressed by ethylene and gibberellin (Loreti *et al*. 2008, Jeong *et al*. 2010, Kwon *et al*. 2011). Previously, we found that BR enhances the JA-induced anthocyanin accumulation (Peng *et al*. 2011). In this work, we found that BR also enhanced the CK-induced anthocyanin biosynthesis. These findings demonstrate that the anthocyanin biosynthesis was also regulated by the interaction of plant hormones.

The CK-induced anthocyanin accumulation was reduced in *psc1* and *dwf4-102* mutants (Table 2)

defective in BR biosynthesis (Nakamoto *et al*. 2006, Ren *et al*. 2009) but increased in WT treated with exogenous BR (Table 3) indicating that BR positively regulated the anthocyanin biosynthesis induced by CK. Since the CK-induced anthocyanin accumulation was also significantly reduced in BR signalling mutant *bri1-4* compared to WT (Table 4) even though a very high amount of endogenous BR accumulates in *bri1-4* mutant (Noguchi *et al*. 1999), we concluded that BR regulated the CK-induced anthocyanin accumulation through BR signalling.

Consistent with a reduction of anthocyanin accumulation induced by CK in *bri1-4* and *dwf4-102* (Tables 2 and 4), the CK-induced expressions of the late anthocyanin biosynthetic genes including *DFR*, *LDOX*, and *UF3GT* were also significantly reduced in both *bri1-4* and *dwf4-102* (Table 5). *Arabidopsis* WD-repeat/ Myb/bHLH complexes including transcription factors *PAP1*, *PAP2*, *EGL3*, *GL3*, and *TTG1* predominantly regulate the expression of the late anthocyanin biosynthetic genes more than the expression of the early anthocyanin biosynthetic genes (Gonzalez *et al*. 2008). In this study, the expressions of transcription factors *PAP1*, *GL3*, and *EGL3* were remarkably induced by CK in WTs but not in *bri1-4* and *dwf4-102* (Table 6). Therefore, we speculate that BR enhanced the CK-induced anthocyanin biosynthesis by up-regulating the late anthocyanin biosynthetic genes including *DFR*, *LDOX*, and *UF3GT*, and this regulation might be mediated by the transcription factors *PAP1*, *GL3*, and *EGL3*.

The anthocyanin biosynthesis is a branch of flavonoids biosynthetic pathways in which the early anthocyanin biosynthetic genes are common to different flavonoids sub-pathways (Koes *et al*. 1994). The early anthocyanin biosynthetic genes *CHS* and *CHI* are considered as defense genes taking part in response to various environmental stimuli, such as low temperature, UV radiation, mechanical wounding, and plant-microbe interactions (Mehdy and Lamb 1987, Jenkins *et al*. 2001, Dao *et al*. 2011). Transcriptions of these genes are elevated to improve plant resistance to abiotic and biotic stresses by accumulation of flavonoids and anthocyanins (Winkel-Shirley 2002, Treutter 2005, Dao *et al*. 2011). CK is able to induce the expression of these genes (Table 5, Deikman and Hammer 1995). BR also have certain links with these genes. For example, a high transcription of *CHS* was shown in BR biosynthetic mutants, such as *det2* and *cpd* (Chory *et al*. 1991, Szekeres *et al*. 1996). In this study, we found the expressions of *CHS* and *CHI* also increased in the BR mutants including *dwf4-102* and *bri1-4* in the absence of BA, although an increase of *CHS* in *bri1-4* was not significant (Table 5). Since the anthocyanin accumulation was reduced in *dwf4-102* and *bri1-4* compared to WT (Tables 2 and 4), it was suggested that the BR regulation of the early anthocyanin biosynthetic genes could mediate the biosynthesis of other flavonoids rather than anthocyanin.

Without the BA treatment, the anthocyanin accumulation also decreased in BR-deficient mutants *psc1* and *dwf4-102* (Table 2) but increased in WT treated with exogenous BR (Table 3) implying that BR itself was able to induce the anthocyanin biosynthesis. However, the anthocyanin accumulation in *bri1-4* mutant was not significantly reduced compared to WT (Table 4). This might be due to the mutants background. Compared to Col-0, Ws-2 accumulates less anthocyanins (Teng *et al*. 2005). In our study, the anthocyanin content in Ws-2 was very low without the CK treatment, less than 50 % of that in Col-0 (Tables 2 and 4). Furthermore, the anthocyanin content induced by CK in Ws-2 was only about 36 % of that in Col-0. Consistent with the anthocyanin accumulation in *dwf4-102* and *bri1-4* mutants (Tables 2 and 4), the expressions of the late anthocyanin biosynthetic genes including *DFR*, *LDOX*, and *UF3GT* were significantly reduced in *dwf4-102* but only slightly decreased in *bri1-4* in the absence of BA (Table 5). Accordingly, without the BA treatment, the relative expressions of the transcription factors *GL3* and *EGL3* tended to be lower in *dwf4-102* than in *bri1-4*, although the decrease of their expressions in both *bri1-4* and *dwf4-102* was not significant (Table 6).

Similarly, anthocyanin content was reduced in BR biosynthesis mutant *dwf1-101* grown under continuous far-red radiation (Luccioni *et al*. 2002), and exogenous BR can stimulate anthocyanin biosynthesis in grape, soybean, and rice (Symons *et al*. 2006, Cevahir *et al*. 2008, Farooq *et al*. 2009, Luan *et al*. 2013). However, BR shows inhibitory effects on anthocyanin accumulation in regenerated *Torenia* shoots (Nagira *et al*. 2006). *Arabidopsis bls1* mutant, which is impaired in radiation- and BR-induced responses as well as in sugar signalling, shows a greater anthocyanin accumulation (Laxmi *et al*. 2004) than respective WT. These seemingly contrary results of BR effects on anthocyanin biosynthesis might be due to species and mutants backgrounds.

In conclusion, BR enhanced the CK-induced anthocyanin biosynthesis in the *Arabidopsis* seedlings by up-regulation of the late anthocyanin biosynthetic genes, and this regulation might be mediated by the transcription factors *PAP1*, *GL3*, and *EGL3*.

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