Transcriptional activation of anthocyanin structural genes in *Torenia* 'Kauai Rose' via overexpression of anthocyanin regulatory transcription factors

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Abstract

This study was conducted to examine the role of the transcription factors (TFs), *RsMYB1* and *mPAP1* together with *B-Peru* (*mPAP1* + *B-Peru*), in regulating anthocyanin biosynthesis in the ornamental torenia (*Torenia fournieri*) cultivar Kauai Rose using *Agrobacterium*-mediated transformation. Expression levels of *RsMYB1* were the highest in the lines RS5 and RS3, followed by RS4, RS2, and RS1, while transcript levels of *mPAP1* + *B-Peru* increased in the order of PB-6 > PB-5 > PB-7 > PB-8 > PB-2. Moreover, transcript levels of the anthocyanin structural genes in transgenic lines were significantly higher than those in wild-type (WT) plants. Anthocyanin structural gene expression was specifically altered by TF overexpression: the highest expression of anthocyanidin synthase (*ANS*) was observed in transgenic lines with *RsMYB1*, while expression of dihydroflavonol-4-reductase (*DFR*) was the highest in lines with *mPAP1* + *B-Peru*. We expect that enhanced expression of these anthocyanin structural genes will improve anthocyanin content in the flowers of transgenic torenia. Moreover, these results indicate that *RsMYB1* and *mPAP1* + *B-Peru* can be exploited as anthocyanin regulatory TFs to enhance anthocyanin content in other horticultural plants.

Keywords Flower color · Genetic transformation · Ornamental plant · Quantitative real-time polymerase chain reaction (qRT-PCR) · Anthocyanin · *RsMYB1* · *mPAP1* · *B-Peru*

Introduction

Flower color is one of the most important characteristics of floricultural plants. Plant breeders in floriculture have focused on how flower colors can be modified using several techniques such as interspecific hybridization, mutagenesis, and plant genetic transformation. Over the past decade,

Agrobacterium-mediated genetic transformation has increasingly been used to modify flower colors, with progress using this method in recent years (Cavallini et al. 2014; Schwinn et al. 2014; Ai et al. 2016a, 2017). Flower colors (ranging from red to blue) are influenced by anthocyanins derived from specific branches of the anthocyanin pathway. The transcription factor (TF) R2R3-MYB alone or in combination with a basic helix–loop–helix (bHLH) TF was shown to regulate the transcript levels of structural genes involved in the anthocyanin biosynthetic pathway in various plant species (Albert et al. 2014; Xie et al. 2014; Ai et al. 2016a; Naing et al. 2018). Recently, Lim et al. (2012) and Ai et al. (2016a) reported that overexpression of R2R3-MYB (*mPAP1*) alone or in combination with the bHLH (*B-peru*) in transgenic tobacco and petunia enhanced anthocyanin production. Similar results were observed in transgenic tobacco, *Arabidopsis*, and petunia by overexpression of *RsMYB1* (Lim et al. 2016; Ai et al. 2017).

Torenia (*Torenia fournieri*) is commonly known as both a bedding and potting plant in the floricultural industry and is frequently used as a model crop in genetic

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transformation studies. Many flower companies and ornamental plant breeders are interested in modifying flower color to expand the existing colors of common torenia cultivars. However, modification of flower colors by conventional breeding may be difficult because of the necessity of enormous time and effort; thus, application of the *Agrobacterium*-mediated genetic transformation method may be useful for modifying flower colors. For example, production of blue-colored flower in rose (Katsumoto et al. 2007) and modification of flower colors in various floricultural crops including torenia have been previously reported (Reviewed by Nishihara and Nakatsuka 2011; Aida et al. 2000; Shikata et al. 2011). However, modification of flower colors in torenia by overexpression of anthocyanin regulatory TFs such as R2R3-MYB or bHLH is still relatively rare, particularly modifications using *mPAP1*, *B-peru*, and *RsMYB1*, which increase anthocyanin production in tobacco, *Arabidopsis*, and petunia (Lim et al. 2012, 2016; Ai et al. 2016a, 2017). Thus, it is necessary to introduce the TFs into torenia via *Agrobacterium*-mediated genetic transformation to verify whether the TFs regulate anthocyanin structural genes involved in the anthocyanin biosynthetic pathways of torenia.

In this study, we developed different transgenic lines of torenia overexpressing *mPAP1 + B-peru* and *RsMYB1*, and the transcript levels of anthocyanin structural genes expressed in the different transgenic lines were evaluated via quantitative real-time polymerase chain reaction (qRT-PCR).

Materials and methods

Plant materials

Seeds of *Torenia* ‘Kauai Rose’ (Syngenta Flowers, Inc., Gilroy, CA, USA) were cultured in MS basal medium for 6 weeks, and leaves from the 6-week-old in vitro seedlings were used for *Agrobacterium*-mediated genetic transformation studies.

Plasmid construction

Agrobacterium tumefaciens strains LBA 4404, harboring a binary vector pB7WG2D containing *mPAP1 + B-Peru* cDNAs, and C58C1 harboring the same vector but containing *RsMYB1* cDNA were used in this study (Supplementary Figs. 1 and 2). The TF genes were under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter. The *bar* gene which confers phosphinothricin (PPT) resistance was used as a selectable marker.

Genetic transformation

Genetic transformation was performed, as described by Naing et al. (2017). Briefly, the leaves of 6-week-old in vitro seedlings were cut into 5 mm² pieces and placed separately in cell suspensions of LBA 4404 harboring *mPAP1 + B-Peru* or C58C1 harboring *RsMYB1* for 30 min. Explants were co-cultivated in the dark for 2 days and then transferred into shoot regeneration medium (MS basal medium + IAA + BA) containing 250 mg⁻¹ Clavamox and 0.5 mg⁻¹ PPT. After 30 days of culture, regenerated shoots were transferred into MS basal medium containing 250 mg⁻¹ Clavamox and 1.0 mg⁻¹ PPT for 21 days, and putative transgenic lines that survived well in the PPT-containing medium were used for molecular analysis. The transformation experiment contained 300 explants. Leaf explants added to the same solution without bacterial cell suspension were used as WT samples.

DNA extraction and PCR analysis

Total genomic DNA was isolated from the leaves of different independent putative transgenic lines (*RsMYB1*: RS1, RS2, RS3, RS4, and RS5; *mPAP1 + B-Peru*: PB2, PB5, PB6, PB7, and PB8, and WT plants) using the HiYield™ genomic DNA mini Kit (Real Biotech Corporation, Taipei, Taiwan). The plasmids based on pB7WG2D containing *mPAP1 + B-Peru* or *RsMYB1* were used as positive controls. The primers and PCR conditions for specific genes are shown in Supplementary Table 1. The reaction products were analyzed via electrophoresis on a 2% (w/v) agarose gel stained with ethidium bromide.

RNA isolation

Total RNA was also isolated from the leaves of the same transgenic lines (*RsMYB1*: RS1, RS2, RS3, RS4, and RS5; *mPAP1 + B-Peru*: PB2, PB5, PB6, PB7, and PB8 and WT plants) using the RNAqueous kit (Ambion Inc., Austin, TX, USA), as performed by Ai et al. (2016b). The purity and concentration of the isolated RNA were measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Analysis of reverse transcription- and quantitative real-time-PCR

Reverse transcription was performed using 1 µg of total RNA and an oligo dT₂₀ primer (ReverTra Ace-α, Toyobo, Osaka, Japan). Expression of the TFs and structural genes (*chalcone synthase* [*CHS*], *flavanone-3-hydroxylase* [*F3H*],

dihydroflavonol-4-reductase [DFR], anthocyanidin synthase [ANS]) involved in anthocyanin biosynthesis in the transgenic lines was confirmed via reverse transcription (RT)-PCR. The reaction products were analyzed via electrophoresis on a 2% (w/v) agarose gel stained with ethidium bromide.

Moreover, the transcript levels of the TFs and structural genes were analyzed via qRT-PCR (Step One Plus Real-time PCR system, Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was carried out in a 20 μ L reaction volume containing 0.5 μ M gene-specific primers and Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), as performed by Ai et al. (2016b). Primers and PCR conditions for the detected genes are shown in Supplementary Table 2. The actin gene was used as a reference gene (Mallona et al. 2010). The analysis was repeated three times.

Statistical analysis

Data were statistically analyzed via analysis of variance using SPSS software version 11.09 (SPSS, Inc., Chicago, IL, USA). Data are presented as the means $\pm$ standard error. Mean separations were determined using Duncan's multiple range test, and significance was determined at a 5% level.

Results

Production of torenia transgenic plants overexpressing *RsMYB1* and *mPAP1 + B-Peru*

To examine the involvement of the TFs (*RsMYB1*, and *mPAP1 + B-peru*) in regulating anthocyanin biosynthesis in the torenia cultivar Kauai Rose, we introduced the TFs into torenia via *Agrobacterium*-mediated genetic transformation. At 4 weeks after genetic transformation, we obtained several putative transgenic lines that survived in selective media containing PPT. We randomly selected five independent transgenic lines transformed with *RsMYB1* (RS1, RS2, RS3, RS4, and RS5) and *mPAP1 + B-peru* (PB2, PB5, PB6, PB7, and PB8) for molecular analysis. The presence of the TFs in each transgenic line was detected via PCR (Fig. 1) and RT-PCR analysis (Fig. 2). Moreover, expression of the anthocyanin structural genes (*CHS*, *F3H*, *DFR*, and *ANS*) in the transgenic lines and WT plants was also detected via RT-PCR analysis (Fig. 3).

Transcriptional analysis of TFs in independent transgenic lines

TF (*RsMYB1*, and *mPAP1 + B-Peru*) expression was clearly detectable (Fig. 2), but to measure expression levels of the TFs among the lines, transcript levels were quantified via

Fig. 1 Amplification of *RsMYB1* (a) and *mPAP1 + B-Peru* (b) in transgenic torenia lines, plasmid (P), and wild-type (WT) plants via PCR

Fig. 2 Detection of *RsMYB1* (a) and *mPAP1 + B-Peru* (b) expression in transgenic torenia lines compared to wild-type (WT) via RT-PCR

qRT-PCR. As expected, the transcript levels of the TFs (*RsMYB1*, and *mPAP1 + B-Peru*) in the transgenic lines significantly differed; RS3 exhibited the highest levels of *RsMYB1*, followed by RS1, RS4 and RS5, and RS2, while levels of *mPAP1 + B-Peru* were found to be highest in PB2 followed by PB7, PB8, PB5, and PB6 (Fig. 4).

Transcriptional analysis of anthocyanin structural genes in transgenic lines

When transcript levels of the anthocyanin structural genes (*CHS*, *F3H*, *DFR*, and *ANS*) in the transgenic lines overexpressing *RsMYB1* or *mPAP1 + B-Peru* were analyzed via qRT-PCR, differential expression of the structural genes was observed in all transgenic lines overexpressing the TFs. Their transcript levels were significantly higher in transgenic lines than in WT. In lines overexpressing *RsMYB1*, structural gene expression levels were highest in RS5 and RS3, followed by RS4, RS2, and RS1 (Fig. 5), while in lines overexpressing *mPAP1 + B-Peru*, the structural gene expression levels were in the order PB6 > PB5 > PB7 > PB8 > PB2 (Fig. 6). Expression of *ANS* was higher in transgenic lines overexpressing *RsMYB1*, but expression of *DFR* was higher in lines overexpressing *mPAP1 + B-Peru*. Overall, transcript levels of the anthocyanin structural genes were significantly enhanced in all transgenic lines overexpressing the TFs (*RsMYB1*, and *mPAP1 + B-Peru*); however, transcript levels of the structural genes were not directly correlated

Fig. 3 Detection of expression of anthocyanin structural genes in transgenic *Torenia* lines overexpressing *RsMYB1* (a), *mPAP1 + B-Peru* (b), and wild-type (WT) plants via RT-PCR

Fig. 4 Comparison of transcript levels of *RsMYB1* (a) and *mPAP1 + B-Peru* (b) expressed in independent transgenic lines. Error bars indicate the standard errors of the mean of three replicates.

Expression levels are shown relative to actin expression. Means with different letters are significantly different (Duncan's multiple range test, $p < 0.05$)

with those of the TFs overexpressed in the transgenic lines. For example, transcript levels of the structural genes in the lines RS5 and PB6, which expressed low transcript levels of *RsMYB1* and *mPAP1 + B-Peru*, respectively, were significantly higher than those in RS1 and PB2, which had higher transcript levels of the TFs.

Discussion

Regulation of anthocyanin production involves MYB TFs alone or in combination with bHLH TFs in several plant species (Quattrocchio et al. 2006; Morita et al. 2006; Nakatsuka

et al. 2008; Lin-Wang et al. 2010; Yamagishi et al. 2010; Park et al. 2011). In addition, enhancement of anthocyanin production in ornamental crops by TF overexpression is well-documented (Zvi et al. 2012; Albert et al. 2014; Cavallini et al. 2014; Schwinn et al. 2014; Naing et al. 2018). Increased expression of anthocyanin structural genes, which are involved in anthocyanin production, via overexpression of *RsMYB1* in different chrysanthemum cultivars has been reported (Naing et al. 2015; Kee et al. 2016). Moreover, overexpression of *RsMYB1* or *mPAP1 + B-Peru* in petunia was also found to enhance anthocyanin pigmentation in vegetative and floral tissues by elevating the transcript levels of structural genes (Ai et al. 2016a, 2017). We

Fig. 5 Comparison of transcript levels of the anthocyanin structural genes (a–d) expressed in wild-type (WT) plants and transgenic lines overexpressing *RsMYB1*. Error bars indicate the standard errors of

the mean of three replicates. Expression levels are shown relative to actin expression. Means with different letters are significantly different (Duncan's multiple range test, $p < 0.05$)

Fig. 6 Comparison of transcript levels of the anthocyanin structural genes (a–d) expressed in wild-type (WT) plants and transgenic lines overexpressing *mPAP1 + B-Peru*. Error bars indicate the standard

errors of the mean of three replicates. Expression levels are shown relative to actin expression. Means with different letters are significantly different (Duncan's multiple range test, $p < 0.05$)

examined whether overexpression of the TFs (*RsMYB1*, *mPAP1 + B-Peru*) in the torenia cultivar Kauai Rose would enhance the transcript levels of the structural genes to modify the color of torenia flowers.

RsMYB1 or *mPAP1 + B-Peru* expression was detected in all transgenic lines analyzed via RT-PCR. However, qRT-PCR showed that the transcript levels of the TFs (*RsMYB1*, *mPAP1 + B-peru*) expressed in all transgenic lines significantly differed [higher expression of *RsMYB1* in RS3 followed by RS1, RS4 and RS5, and RS2 (Fig. 4a), and that of *mPAP1 + B-Peru* in PB2 followed by PB7, PB8, PB5, and PB6 (Fig. 4b)]. These results are in agreement with the findings of Ai et al. (2016a, 2017) and Kee et al. (2016), who also observed differential expression of the same TFs in independent transgenic petunia and *chrysanthemum* lines. Interestingly, the transcript levels of the TFs were not directly correlated with transcriptional activation of the structural genes. This finding is consistent with the results of Cho et al. (2016), because they reported that PtrMYB119 functions as a transcriptional activator of anthocyanin structural genes in poplar, but transcript levels of the TF expressed in the transgenic lines are not directly correlated with those of the structural genes. Regardless, the transcript levels of structural genes in the transgenic lines were significantly higher than those in WT plants.

Among the structural genes, high elevation of late biosynthesis genes such as *DFR* and *ANS* was notable in our transgenic lines. Elevation of late biosynthesis genes by overexpression of the MYB TFs alone or in combination with bHLH has been reported previously (Schwinn et al. 2014; Naing et al. 2018). In recent decades, the importance of anthocyanin structural genes in anthocyanin accumulation in torenia has been reported (Aida et al. 2000; Suzuki et al. 2000; Fukusaki et al. 2004; Nakamura et al. 2006). Suzuki et al. (2000) reported that transcriptional suppression of *CHS* or *DFR* in *Torenia hybrida* 'Summerwave' changed the flowers from blue to white or partly white. Similarly, Aida et al. (2000) and Fukusaki et al. (2004) showed that suppression of *CHS* or *DFR* changed the blueness of the flower to pale or white. In addition, Nakamura et al. (2006) suggested that suppression of *ANS* by RNAi in *Torenia hybrida* resulted in the production of white flowers. Moreover, overexpression of *F3H* reddened the flower colors in torenia (Ueyama et al. 2002).

In our study, significant elevation of the above-mentioned structural genes was observed in transgenic lines overexpressing *RsMYB1* or *mPAP1 + B-Peru* compared to WT plants. However, it can be expected that anthocyanin accumulation in lines overexpressing *mPAP1 + B-Peru* would be higher than those overexpressing *RsMYB1*, because the transcript levels of the anthocyanin structural genes, except *ANS*, were higher in transgenic lines overexpressing *mPAP1 + B-Peru* compared to those expressing *RsMYB1*

(Figs. 5, 6). Co-expression of MYB (*mPAP1*) and bHLH (*B-Peru*) resulted in more enhanced expression of anthocyanin structural genes than expression of *RsMYB1* alone. Enhanced expression of anthocyanin structural genes via co-expression of MYB and bHLH has also been reported in several plant species (Lloyd et al. 1992; Quattrocchio et al. 1998; Butelli et al. 2008). The transcriptional activation of the anthocyanin structural genes in the transgenic lines in this study is similar to that reported in the previous studies (Ai et al. 2016a, 2017); those studies reported that overexpression of *mPAP1 + B-Peru* and *RsMYB1* in petunia enhanced the anthocyanin content via transcriptional activation of the anthocyanin structural genes (Ai et al. 2016a, 2017). Therefore, we expect that increased anthocyanin will be visually observed in the flowers of transgenic torenia overexpressing the TF genes.

Conclusions

The results of this study demonstrate that overexpression of *RsMYB1* or *mPAP1 + B-Peru* in a torenia cultivar significantly enhances the transcript levels of anthocyanin structural genes (*CHS*, *F3H*, *DFR*, and *ANS*) compared to WT plants. In general, transcript levels of the structural genes (except *ANS*) were higher in plants overexpressing *mPAP1 + B-Peru* than those overexpressing *RsMYB1*, whereas stronger induction of *ANS* by *RsMYB1* and of *DFR* by *mPAP1 + B-Peru* was observed. Thus, overexpression of these TFs can be used to modify flower color of torenia by enhancing the anthocyanin content. Moreover, these results provide insight into the role of the TFs in regulating anthocyanin structural genes, which will be helpful for plant breeders working to modify flower colors or improve anthocyanin content in fruits and vegetables.

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Author contributions AHN and XJ designed the study, conducted the experiments, and wrote the manuscript. CKK supervised experiments at all stages and performed critical revisions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest There are no conflicts of interest.

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