Scientia Horticulturae 240 (2018) 411–416

Contents lists available at ScienceDirect

Scientia Horticulturae

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

Ectopic expression of the R2R3-MYB gene from *Tricyrtis* sp. results in leaf color alteration in transgenic *Pelargonium crispum*

Atsushi Kanemaki[a,1], Masahiro Otani[a,1], Miho Takano[a], Takuo Fujimoto[a], Hiroaki Okuhara[b], Toshikazu Nomizu[b], Masayoshi Kondo[b], Hitoshi Kobayashi[b], Fumi Tatsuzawa[c], Masaru Nakano[a,*]

[a] Graduate School of Science and Technology, Niigata University, 2-8050 Ikarashi, Nishi-ku, Niigata 950-2181, Japan
[b] Niigata Agricultural Research Institute, 857 Nagakura, Nagaoka 940-0826, Japan
[c] Faculty of Agriculture, Iwate University, 3-18-8 Ueda, Morioka 020-8550, Japan

ARTICLE INFO

Keywords:
Anthocyanin
Colored-leaf plant
Flavonoid biosynthetic pathway
MYB transcription factor
Ornamental plant

ABSTRACT

R2R3-MYB transcription factors are known to activate the flavonoid biosynthetic pathway. In the present study, R2R3-MYB gene isolated from the liliaceous ornamental plant *Tricyrtis* sp. (*TrMYB1*) under the control of the cauliflower mosaic virus 35S promoter was introduced into *Pelargonium crispum* via *Agrobacterium*-mediated transformation in order to alter leaf color phenotype. Ten independent transgenic plants have been obtained, and they could be classified into three types according to the leaf color phenotype: six transgenic plants had deep yellowish-green leaves as non-transgenic plants (Type I); two had deep red-purple leaves (Type II); and two had deep red leaves (Type III). Spectrophotometric analysis showed that the amount of total anthocyanins significantly increased in leaves of Type II and Type III transgenic plants compared with non-transgenic and Type I transgenic plants. In addition, several anthocyanins were newly produced in leaves of Type II and Type III transgenic plants as revealed by high performance liquid chromatography analysis. Quantitative real-time reverse transcription-polymerase chain reaction analysis showed that *TrMYB1* expression level correlated with the degree of leaf color alteration. Our results indicate the validity of genetic transformation with *TrMYB1* for producing colored foliage in heterologous ornamental plants.

1. Introduction

Leaf color is one of the attractive traits for ornamental plants, and plants with colored foliage often called “colored-leaf plants”. In most colored-leaf plants, such as coleus (*Solenostemon* spp.), *Heuchera* spp. and ornamental kale (*Brassica oleracea* var. *acephala*), anthocyanins are accumulated in leaf cells, resulting in red, purple or violet foliage. Anthocyanins are synthesized through the flavonoid biosynthetic pathway, and the biochemistry and enzymology of this pathway are well established (Forkmann, 1991; Koes et al., 2005). Genes for flavonoid biosynthetic enzymes have so far been isolated from various plant species, and successful flower color alteration by genetic transformation using these genes has already been reported as follows: *Petunia hybrida* with overexpressed foreign dihydroflavonol 4-reductase (DFR) gene (Oud et al., 1995); *Rosa hybrida* with suppressed endogenous DFR gene and overexpressed foreign flavonoid 3′,5′-hydroxylase (F3′5′H) and DFR genes simultaneously (Katsumoto et al., 2007); *Cyclamen persicum* with suppressed endogenous F3′5′H gene (Boase et al., 2010); *Gentiana triflora* × *G. scabra* with suppressed both endogenous 5,3′-aromatic acyltransferase (5/3′AT) and F3′5′H genes (Nakatsuka et al., 2010); *Torenia hybrida* with suppressed endogenous flavonoid 3′-hydroxylase (F3′H) and F3′5′H genes and overexpressed foreign DFR gene, simultaneously (Nakamura et al., 2010); *Tricyrtis* sp. with suppressed endogenous chalcone synthase (CHS) gene (Kamiishi et al., 2012); *Chrysanthemum morifolium* with overexpressed foreign F3′5′H gene (Noda et al., 2013). Although ectopic expression of the flavonoid biosynthetic enzyme genes is assumed to alter leaf color in addition to flower color, production of colored-leaf plants has not yet been achieved by genetic transformation with a single flavonoid biosynthetic enzyme gene due to the lack of expression of multiple flavonoid biosynthetic enzyme genes in leaves (Bradley et al., 1998; Nakatsuka et al., 2005). Thus, in order to alter leaf color phenotype, it is necessary to introduce or upregulate multiple genes encoding flavonoid biosynthetic enzymes simultaneously.

R2R3-MYB transcription factor forms a trimeric activator complex (MBW complex) with basic helix-loop-helix (bHLH) and WD40 repeat

* Corresponding author at: Faculty of Agriculture, Niigata University, 2-8050 Ikarashi, Niigata 950-2181, Japan.
E-mail address: mnakano@agr.niigata-u.ac.jp (M. Nakano).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.scienta.2018.06.029
Received 6 April 2018; Received in revised form 1 June 2018; Accepted 13 June 2018

(WDR) proteins, which generally induces anthocyanin accumulation through transcriptional upregulation of multiple genes for flavonoid biosynthetic enzymes (Koes et al., 2005). R2R3-MYB genes have so far been isolated from various plant species, and heterogeneous expression of these genes in some model plants such as *Arabidopsis thaliana* (Borevitz et al., 2000) and *Nicotiana tabacum* (Chen et al., 2017; Deluc et al., 2006; Huang et al., 2013; Mooney et al., 1995) has led to obvious anthocyanin accumulation in the whole body. Thus, genetic transformation using the R2R3-MYB genes may be an effective mean to produce "colored-leaf plants". However, there have been only a few studies on leaf color alteration by genetic transformation with the R2R3-MYB genes in ornamental plants such as *P. hybrida* (Bradley et al., 1998), *Gerbera hybrida* (Laitinen et al., 2008), *Ficus lyrata* (Zhao et al., 2013), and *R. hybrida* (Zvi et al., 2012).

Pelargonium spp., generally called "geraniums", are economically important ornamental plants belonging to Geraniaceae. Among *Pelargonium* spp., *P. crispum*, which has lemon-scented foliage and beautiful flowers, is cultivated worldwide for pot and garden uses as well as production of aroma oils (Lim, 2014). In *P. crispum*, there are few cultivars with colored foliage at present, production of colored-leaf plants is desired for enhancing their horticultural and economical value. Therefore, in the present study, we examined production and characterization of transgenic *P. crispum* plants ectopically expressing the R2R3-MYB gene isolated from the liliaceous ornamental plant *Tricyrtis* sp. (*TrMYB1*) (Otani et al., 2018).

2. Materials and methods

2.1. Plant material and plantlet culture

Potted plants of *Pelargonium crispum* cv. Angel Eyes Viola were used in the present study. They were cultivated in the greenhouse, where the night/day temperatures were 5–20 °C / 10–30 °C, respectively, depending on the season and weather.

Vigorously growing stems were harvested from potted plants and surface-sterilized with 70% ethanol for 1 min followed by with a sodium hypochlorite solution (1% active chlorine) containing a drop of Tween 20 for 10 min. After three rinses with sterilized distilled water, nodal segments (ca. 10 mm in length) were placed on a plantlet culture medium [half-strength MS medium (Murashige and Skoog, 1962) containing 1 mg L^{-1} indole-3-butyric acid (IBA), 20 g L^{-1} sucrose, and 3 g L^{-1} gellan gum, pH 5.8]. Axillary bud-derived shoots were subcultured every two months by transferring nodal segments (ca. 10 mm in length) to fresh medium of the same composition. Cultures were maintained at 25 °C under a 16 h photoperiod with fluorescent light (35 µmol m^{-2} s^{-1}).

2.2. Agrobacterium-mediated production of transgenic plants

A. tumefaciens strain EHA101/pIG-*TrMYB1* was used for transformation. The T-DNA region of the binary vector pIG-*TrMYB1* contained *TrMYB1* (accession number AB856412 in the GenBank/EMBL/DDBJ databases) under the control of the cauliflower mosaic virus (CaMV) 35S promoter, the neomycin phosphotransferase II (*NPTII*) gene under the control of the nopaline synthase (NOS) promoter, and the hygromycin phosphotransferase (*HPT*) gene under the control of the CaMV35S promoter (Fig. S1). *A. tumefaciens* strain EHA101/pIG121-Hm (accession number AB489142 in the GenBank/EMBL/DDBJ databases) was also used in the present study as a vector control.

Production of transgenic *P. crispum* plants was performed as previously described for *P. zonale* (syn. *P.* × *hortorum*) and *P. capitatum* (Hassanein et al., 2005) with several modifications. Petiole segments (ca. 5 mm in length) were collected from plantlet cultures two months after subculture and precultured on a co-cultivation medium [half-strength MS medium containing 0.1 mg L^{-1} 1-naphthaleneacetic acid (NAA), 1 mg L^{-1} 6-benzyladenine (BA), 1 mg L^{-1} zeatin, 50 mg L^{-1} acetosyringone (AS), 0.5 g L^{-1} 2-(N-morpholino) ethanesulfonic acid (MES), 30 g L^{-1} sucrose, and 2 g L^{-1} gellan gum, pH 5.8] at 25 °C in the dark for 3 days. Cultured *Agrobacterium* cells were suspended to an OD_{600} = 0.8–1.2 in an inoculation medium (half-strength MS medium containing 50 mg L^{-1} AS, 0.5 g L^{-1} MES, and 30 g L^{-1} sucrose, pH 5.8). Precultured petiole segments were immersed in the bacterial suspension for 20 min with gentle shaking. After blot-drying on sterilized filter papers, petiole segments were transferred to the co-cultivation medium and incubated at 25 °C in the dark for 3 days. Co-cultivated segments were then transferred to a selection medium (half-strength MS medium containing 0.2 mg L^{-1} NAA, 0.5 mg L^{-1} BA, 0.5 mg L^{-1} zeatin, 50 mg L^{-1} kanamycin, 20 mg L^{-1} meropenem, 0.5 g L^{-1} MES, 30 g L^{-1} sucrose, and 2 g L^{-1} gellan gum, pH 5.8) and subcultured every two weeks to fresh medium of the same composition at 25 °C under a 16 h photoperiod with fluorescent light (35 µmol m^{-2} s^{-1}). Adventitious shoots (ca. 10 mm in length) regenerated within two months were isolated from co-cultivated segments, transferred for rooting to the plantlet culture medium supplemented with 20 mg L^{-1} meropenem, and cultured under the same conditions.

The presence of the transgene in regenerated plantlets was confirmed using PCR analysis with a *TrMYB1*-specific primer set, TrMYB1 RT-Fw (5′-TGA GCA GCA CCA AGA AGA TG-3′) and TrMYB1 RT-Rev (5′-TGC AAA ATC ATA CTA ACC CCA GAG-3′).

After acclimatization, transgenic plantlets were transplanted to pots and cultivated in a growth chamber at 25 °C under a 16 h photoperiod with fluorescent light (35 µmol m^{-2} s^{-1}). After six months of cultivation, morphological, biochemical and molecular characterizations were performed during the flowering season.

2.3. Leaf color characterization

Leaf color characterization was performed using leaf blades other than vines. Initially leaf color was investigated visually with the aid of the JHS Color Chart (Japan Horticultural Plant Standard Color Chart, 1984) and expressed using Inter-Society Color Council, National Bureau of Standard color name as well as JHS Color Chart number according to Kuwayama et al. (2005). Then, lightness (L^*) and two chromatic components a^* and b^* of the CIE$L^*a^*b^*$ color coordinates (Gonnet, 1995) of leaves were measured using the Color Reader (CR-400; Konica Minolta Sensing Inc., Tokyo, Japan). L^* values indicate lightness (0 = black, 100 = white). The a^* and b^* values which shift from negative to positive values indicate to shift from green to red and from blue to yellow, respectively.

2.4. Measurement of anthocyanin contents

Total anthocyanins were extracted from 100 mg of fresh leaves with a methanol-HCl method according to Rabino and Mancinelli (1986). Absorption of the extracts at 530 and 657 nm wavelength was measured using a spectrophotometer (Ultrospec 3000; GE healthcare UK Ltd., Little Chalfont, England). Relative amount of total anthocyanins was calculated with the formula, $(A_{530} - 0.25 \times A_{657})\ M^{-1}$, where A_{530} and A_{657} are the absorbances at the indicated wavelengths and M (g) is the mass of the plant material used for extraction. Each measurement was performed in triplicate.

2.5. High performance liquid chromatography (HPLC) analysis of anthocyanidins

Anthocyanidins were extracted from 300 mg of fresh leaves and analyzed by HPLC according to Kamiishi et al. (2012). Anthocyanidin extracts from petals of *Torenia fournieri* were used as anthocyanidin standard and five peaks detected in the present study were identified.

2.6. Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Transgene transcripts in young leaves were quantified by real-time RT-PCR analysis. Total RNA was extracted according to the IHBT protocol (Ghawana et al., 2011) and then treated with DNaseI (Life Technologies, CA, USA) according to the manufacturer's instructions. For cDNA synthesis, 500 ng of the total RNA was reverse-transcribed in a total volume of 20 µL using the PrimeScript™ RT reagent Kit, Perfect Real Time (Takara, Shiga, Japan) according to the manufacturer's protocol. Real-time RT-PCR analysis was performed using SYBR® Premix Ex Taq™II (Takara, Shiga, Japan) on the DNA Engine Opticon System (MJ Research, Waltham, MA, USA) as previously described (Otani et al., 2016). The primer sets used were the above-mentioned *TrMYB1*-specific primer set, TrMYB1 RT-Fw and TrMYB1 RT-Rev; and an Actin 7-like protein gene (accession number FJ834457 in GenBank/EMBL/DDBJ databases)-specific primer set, Actin RT-Fw (5′-AGG CTG GAT TTG CTG GTG ATG-3′) and Actin RT-Rev (5′-GCC TTG GGG TTG AGA GGT G-3′). Each PCR was performed in triplicate under the following conditions: 5 s at 95 °C, 30 s at 60 °C, and plate read (detection of fluorescent product) for 45 cycles. To characterize the PCR products, a melting curve analysis was performed by slowly raising the temperature from 70 to 95 °C, with fluorescence data at 1.0 °C intervals (Ririe et al., 1997). The relative amount of *TrMYB1* transcripts was calculated using the comparative cycle threshold method, and results normalized to the Actin 7-like protein gene.

3. Results

3.1. Production and leaf color phenotype of transgenic P. crispum

Three days after the onset of co-cultivation with *Agrobacterium*, red pigmentation was observed at the cut edges of petiole segments, which may be resulted from transient expression of *TrMYB1* (Fig. S2B). No red pigmentation was observed in the vector control (Fig. S2A). Two weeks after culture on the selection medium, adventitious shoots were produced from co-cultivated petiole segments. During culture in the dark, both white and red shoots were observed (Fig. S2C). Kanamycin-resistant shoots were isolated and transferred to the meropenem-containing plantlet culture medium, on which the shoots developed into plantlets within two weeks. Plantlets developed from red shoots also had red leaves (Fig. S2D).

A total of ten plantlets were confirmed to be transgenic by PCR analysis (Fig. 1) and termed MYB1 lines. Morphological characterization was performed six months after cultivation in pots during the flowering season (Fig. 2; Table S1). MYB1 lines could be classified into the following three types according to the degree of leaf color alteration. Type I MYB1 lines (MYB1-1, -4, -5, -6, -7, -8) had deep yellowish-green leaves as wild-type plants (Fig. 2A and C). Leaves of Type II (MYB1-2, -9) and Type III (MYB 1-3, -10) MYB1 lines were deep red-purple and deep red, respectively (Fig. 2A and C). In both Type II and Type III MYB1 lines, leaf vines showed strong pigmentation (Fig. 2C). Flowers of Type II and Type III MYB1 lines showed slightly deeper colors and larger purple spots compared with those of wild-type plants and Type I MYB1 lines (Fig. 2B). No apparent differences in growth and anthesis were observed between three types of transgenic lines and wild-type plants. No apparent sensuous difference in leaf scent was also observed.

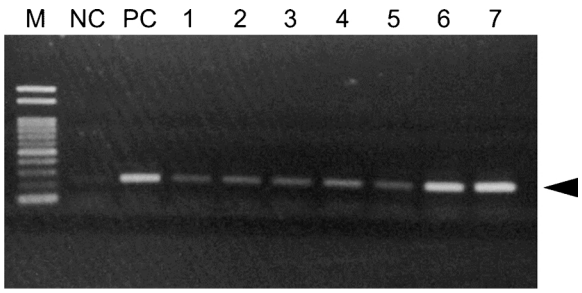

Fig. 1. PCR analysis for detecting the transgene (*TrMYB1*) in putative transgenic plantlets of *Pelargonium crispum*. Lane M, 100-bp ladder, Lane NC, non-transgenic plantlets as a negative control, Lane PC, binary vector pIG-*TrMYB1* as a positive control, Lanes 1–7 independent putative transgenic plantlets. Arrowhead indicates the desired 226 bp fragment.

Distributions based on two chromatic components, a^* and b^* values in each strain were distinctly different on the CIE$L^*a^*b^*$ color coordinates (Fig. S3; Table S1). Leaves of Type III MYB1 lines showed lower L^* (18.8–19.4) and b^* values (5.9–6.3) and higher a^* values (7.6–8.7) than those of wild-type plants and Type I MYB1 lines (L^* = 21.2–24.7; a^* = -12.4–-11.4; b^* = 16.1–12.4). For leaves of Type III MYB1 lines, the degree of changes in a^* and b^* values was much higher than Type II MYB1 lines, although L^* value was almost the same as Type II MYB1 lines.

3.2. Anthocyanin and anthocyanidin analyses in transgenic P. crispum

Leaves of wild-type plants, and Type I (MYB1-1), Type II (MYB1-2) and Type III (MYB1-3) MYB1 lines were used for quantification of total anthocyanins (Fig. 3). Anthocyanin extracts from MYB1-2 and MYB1-3 showed purple and red colors, respectively (Fig. 3A). MYB1-1 showed only a little amount of total anthocyanins, and no significant difference in the amount was observed between MYB1-1 and wild-type plants. MYB1-2 and MYB1-3 showed significantly higher amounts of total anthocyanins than wild-type plants and MYB1-1. In addition, the amount in MYB 1-3 was approximately 3.7-fold higher than MYB1-2 (Fig. 3B).

Fig. 4 shows HPLC profiles of anthocyanidins in leaves of wild-type plants, MYB1-1, MYB1-2 and MYB1-3. No peaks of anthocyanidins were detected in leaves of both wild-type plants and MYB1-1. On the other hand, nine peaks were detected in leaves of both MYB1-2 and MYB1-3. Leaves of MYB1-2 and MYB1-3 mainly contained peonidin 3,5-diglucoside (peak 4) and malvidin 3,5-diglucoside (peak 5). The signal strength of the peaks of these two anthocyanidins in MYB1-3 was much higher than MYB1-2, although no apparent differences were observed in the other seven peaks (peak 1–3 and 6–9) between MYB1-2 and MYB1-3.

3.3. Real-time RT-PCR analysis for TrMYB1 expression in transgenic P. crispum

Fig. 5 shows the relative amount of *TrMYB1* transcripts in young leaves of wild-type plants, and Type I (MYB1-1), Type II (MYB1-2) and Type III (MYB1-3) MYB1 lines. *TrMYB1* transcripts were observed in MYB1-2 and MYB1-3, but not in wild-type plants and MYB1-1. The relative amount of *TrMYB1* transcripts in MYB1-3 was significantly higher than wild-type plants and MYB1-1 and approximately two-fold higher than in MYB1-2.

4. Discussion

In the present study, we successfully produced transgenic *P. crispum* plants ectopically expressing *TrMYB1* by *Agrobacterium*-mediated transformation. Although genetic transformation has already been reported for some *Pelargonium* spp. such a *P.* × *domesticum* (Boase et al., 1996), *P. capitatum* (Hassanein et al., 2005), *P. zonal* (syn. *P.* × *hortorum*) and *P. peltatum* (Hassanein et al., 2005; García-Sogo et al., 2012), and *P. graveolens* (Singh et al., 2017), to the best of our knowledge, this is the first report of genetic transformation of *P. crispum*.

Among transgenic plants obtained, Type II and Type III MYB1 lines had deep red-purple and deep red leaves, respectively (Fig. 2; Table S1). Leaves of Type II and Type III MYB1 lines showed increased a^* values

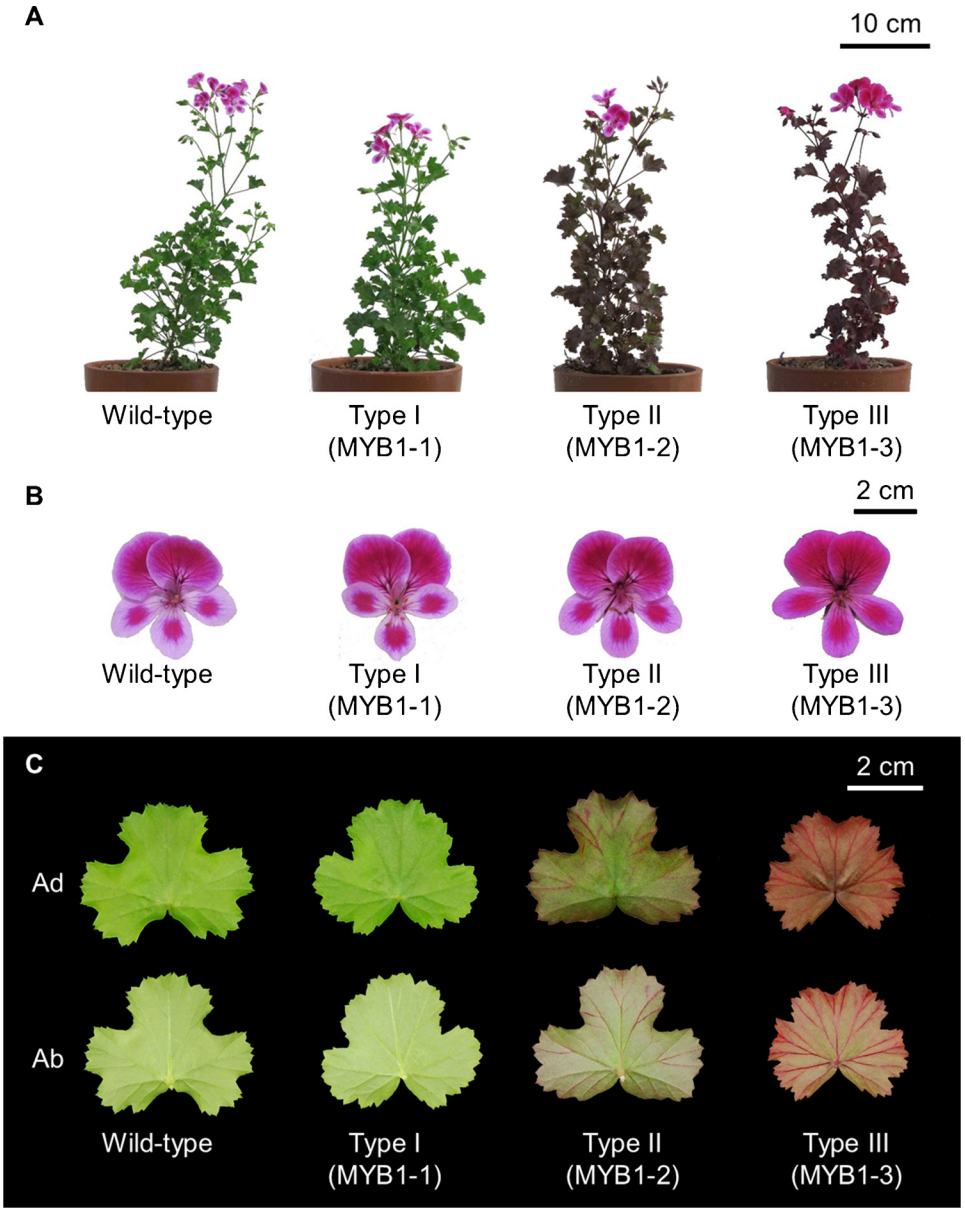

Fig. 2. Morphological characterization of transgenic *Pelargonium crispum* plants. (A) Plants during the flowering season. (B) Flowers. (C) Adaxial (upper, Ad) and abaxial (lower, Ab) sides of leaves.

and decreased b^* values compared with wild-type plants and Type I MYB1 lines, and the degree of changes in a^* and b^* values was much higher in leaves of Type III than Type II MYB1 lines (Fig. S3; Table S1). These results indicate that leaf color turned into reddish and bluish from greenish-yellow in Type II and Type III MYB1 lines, respectively (Fig. S3; Table S1). Similar leaf color phenotypes have been reported for transgenic *P. hybrida* (Bradley et al., 1998), *G. hybrida* (Laitinen et al., 2008), *R. hybrida* (Zvi et al., 2012) and *F. lyrata* (Zhao et al., 2013) ectopically expressing the R2R3-MYB genes. Dasgupta et al. (2017) reported that transgenic *N. tabacum* plants ectopically expressing the R2R3-MYB genes showed red, purple or pink colored-leaves due to anthocyanin accumulation and these leaves showed increased a^* values and decreased b^* values compared with wild-type plants. Similar observations were obtained in the present study for Type II and Type III MYB1 lines. In Type II and Type III MYB1 lines, leaf vines showed strong pigmentation compared with other leaf parts (Fig. 2C). The CaMV35S promoter used in the present study generally has stronger transcriptional activities in vascular tissues (Terada and Shimamoto, 1990; Yang and Christou, 1990). Thus, strong pigmentation in leaf veins in Type II and Type III MYB1 lines may be caused by the property of the CaMV35S promoter. Although stunted and delayed growth has been reported for some transgenic plants of *N. tabacum* (Huang et al., 2013) and *P. hybrida* (Bradley et al., 1998) overexpressing the R2R3-MYB genes under the control of the CaMV35S promoter, Type II and Type III MYB1 lines in the present study showed normal growth and anthesis.

The expression level of *TrMYB1* was positively correlated with the relative amount of total anthocyanins in leaves of transgenic plants (Figs. 3 and 5). *TrMYB1* transcripts were detected in Type II and Type III MYB1 lines, whereas not in Type I MYB1 lines (Fig. 5). Type I MYB1 lines had deep yellowish-green leaves without anthocyanin accumulation as wild-type plants (Figs. 2 and 3; Table S1), and there were no differences in L^*, a^* and b^* values between wild-type plants and Type I MYB1 lines (Fig. S3). No leaf color alteration in Type I MYB1 lines may be caused by silencing of transgene expression. Position effect and co-suppression are most commonly responsible for transgene silencing in plants (Matzke et al., 1989; Matzke and Matzke, 1998; Napoli et al., 1990).

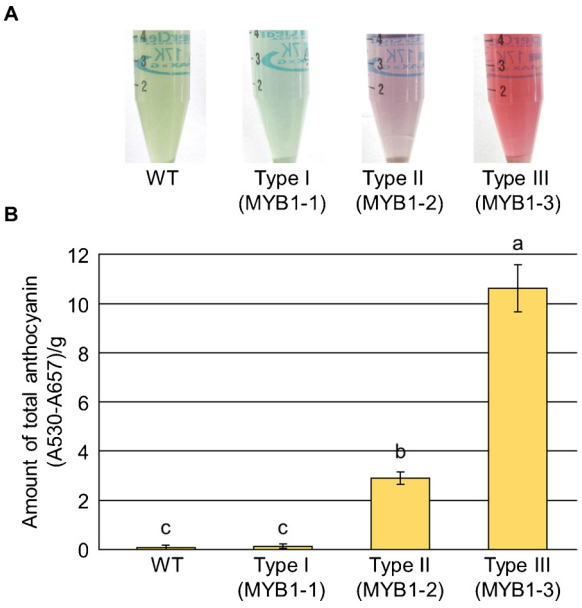

Fig. 3. Anthocyanin contents in leaves of transgenic *Pelargonium crispum* plants. (A) Extracted solutions from leaves and (B) relative amount of total anthocyanins of wild-type plants (WT), and Type I (MYB1-1), Type II (MYB1-2) and Type III (MYB1-3) MYB1 lines. Values with different letters are significantly different at the 0.1 level with the Tukey-Kramer's test.

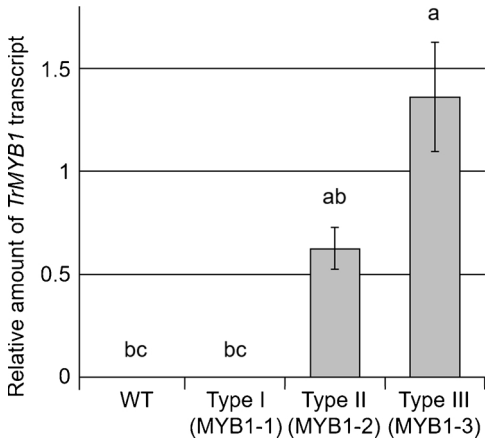

Fig. 5. Real-time RT-PCR analysis for *TrMYB1* expression in leaves of transgenic *Pelargonium crispum* plants. Relative amounts of *TrMYB1* transcripts in wild-type plants (WT), and Type I (MYB1-1), Type II (MYB1-2) and Type III (MYB1-3) MYB1 lines were normalized to the Actin 7-like protein gene. Values with different letters are significantly different at the 0.05 level with the Tukey–Kramer's test.

R2R3-MYB transcription factors act as a trimeric activator complex with bHLH and WDR (Koes et al., 2005). In the model legume plant *Medicago sativa*, ectopic expression of a heterologous R2R3-MYB gene from *A. thaliana* (*AtPAP1*) never induced anthocyanin accumulation, although transgenic plants ectopically expressing an own R2R3-MYB gene (*MtLAP1*) accumulated anthocyanins in the whole body (Peel et al., 2009). This result suggests that R2R3-MYB transcription factors might have species specificity for MBW complex formation. On the other hand, in the present study, ectopic expression of *TrMYB1* in transgenic *P. crispum* resulted in anthocyanin accumulation in leaves. This result indicates that a heterologous R2R3-MYB from the liliaceous monocotyledon *Tricyrtis* sp. can form the active complex with bHLH and WDR of the geraniaceous dicotyledon *P. crispum*. In addition, our result indicates that endogenous genes for bHLH and WDR may be expressed constantly in leaves of *P. crispum*.

Recently, validity of the R2R3-MYB gene as a visual selection marker of transgenic tissues has been examined in order to develop antibiotic-free transformation systems (Kortstee et al., 2011; Saika et al., 2011; Naing et al., 2015). In the present study, some adventitious shoots produced on the kanamycin-containing selection medium showed red pigmentation, and these red shoots subsequently developed into plantlets with red leaves (Fig. S2). All of them were verified to be transgenic by PCR analysis and classified into Type II or Type III MYB1

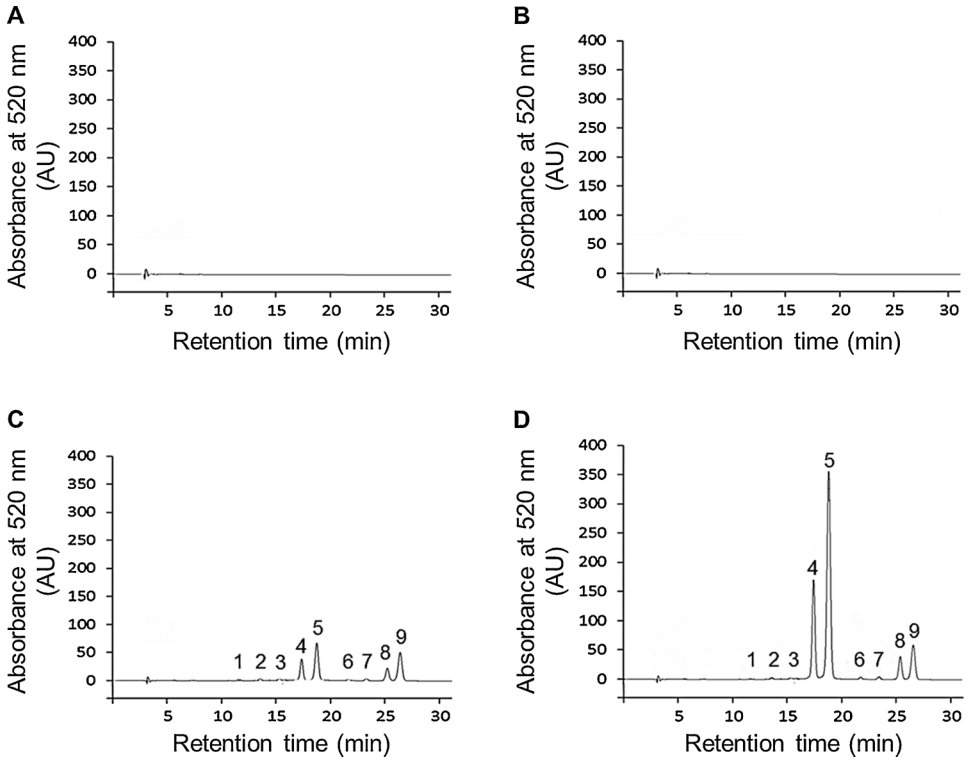

Fig. 4. HPLC profiles of anthocyanidins in leaves of transgenic *Pelargonium crispum* plants. (A) wild-type plants, (B) Type I MYB1 line (MYB1-1), (C) Type II MYB1 line (MYB1-2), and (D) Type III MYB1 line (MYB1-3). Chromatograms' peak identity: 1, delphinidin 3,5-diglucoside; 2, cyanidin 3,5-diglucoside; 3, petunidin 3,5-diglucoside; 4, peonidin 3,5-diglucoside; 5, malvidin 3,5-diglucoside; 6–9, unknowns.

lines. These results indicate that transgenic *P. crispum* plants can partly be screened by using *TrMYB1* as a visual selection marker gene. Although transgenic plants expressing the R2R3-MYB gene under the control of the CaMV35S promoter generally show anthocyanin pigmentation in the whole body, this problem can be overcome by using tissue-specific or drug-inducible promoters, or by removing the R2R3-MYB selection marker gene from the host genome using the Cre/*loxP* site-specific recombination system after selection process (Wang et al., 2005; Dutt et al., 2018).

In conclusion, we successfully produced colored-leaf *P. crispum* plants by genetic transformation using the R2R3-MYB gene from *Tricyrtis* sp. These transgenic plants are horticulturally attractive since they have ornamental values during the non-flowering season. The results obtained in the present study indicate the applicability of the R2R3-MYB gene derived from monocotyledonous plants for molecular breeding of dicotyledonous plants. Thus, we are now examining production of colored-leaf plants in a wide range of ornamental plant species by genetic transformation with *TrMYB1*.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for profit sectors.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.scienta.2018.06.029.

References

Boase, M.R., Deroles, S.C., Winnefield, C.S., Butcher, S.M., Borst, N.K., Butler, R.C., 1996. Genetic transformation of regal pelargonium (*Pelargonium* × *domesticum* 'Dubonnet') by *Agrobacterium tumefaciens*. Plant Sci. 121, 47–61.

Boase, M.R., Lewis, D.H., Davies, K.M., Marshall, G.B., Patel, D., Schwinn, K.E., Deroles, S.C., 2010. Isolation and antisense suppression of *flavonoid 3',5'-hydroxylase* modifies flower pigments and colour in cyclamen. BMC Plant Biol. 10, 107.

Borevitz, J.O., Xia, Y., Blount, J., Dixon, R.A., Lamb, C., 2000. Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell 12, 2383–2394.

Bradley, J.M., Davies, K.M., Deroles, S.C., Bloor, S.J., Lewis, D.H., 1998. The maize *Lc* regulatory gene up-regulates the flavonoid biosynthetic pathway of *Petunia*. Plant J. 13, 381–392.

Chen, K., Liu, H., Lou, Q., Liu, Y., 2017. Ectopic expression of the grape hyacinth (*Muscari armeniacum*) R2R3-MYB transcription factor gene, *MaAN2*, induces anthocyanin accumulation in tobacco. Front. Plant Sci. 8, 965.

Dasgupta, K., Thilmony, R., Stover, E., Oliveira, M.L., Thomson, J., 2017. Novel R2R3-MYB transcription factors from *Prunus americana* regulate differential patterns of anthocyanin accumulation in tobacco and citrus. GM Crops Food 8, 85–105.

Deluc, L., Barrieu, F., Marchive, C., Lauvergeat, V., Decendit, A., Richard, T., Carde, J.P., Mérillon, J.M., Hamdi, S., 2006. Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. Plant Physiol. 140, 499–511.

Dutt, M., Zambon, F.T., Erpen, L., Soriano, L., Grosser, J., 2018. Embryo-specific expression of a visual reporter gene as a selection system for citrus transformation. PLoS One 13, e0190413.

Forkmann, G., 1991. Flavonoids as flower pigments: the formation of the natural spectrum and its extension by genetic engineering. Plant Breed. 106, 1–26.

García-Sogo, B., Pineda, B., Roque, E., Antón, T., Atarés, A., Borja, M., Beltrán, J.P., Moreno, V., Cañas, L.A., 2012. Production of engineered long-life and male sterile *Pelargonium* plants. BMC Plant Biol. 12, 156.

Ghawana, S., Paul, A., Kumar, H., Kumar, A., Singh, H., Bhardwaj, P.K., Arti, R., Singh, R.S., Raizada, J., Singh, K., Kumar, S., 2011. An RNA isolation system for plant tissues rich in secondary metabolites. BMC Res. Notes 4, 85.

Gonnet, J.F., 1995. A colorimetric look at the RHS Chart – Perspectives for an instrumental determination of colour codes. J. Hortic. Sci. 70, 191–206.

Hassanein, A., Chevreau, E., Dorion, N., 2005. Highly efficient transformation of zonal (*Pelargonium* × *hortorum*) and scented (*P. capitatum*) geraniums via *Agrobacterium tumefaciens* using leaf discs. Plant Sci. 169, 532–541.

Huang, W., Sun, W., Lv, H., Luo, M., Zeng, S., Pattanaik, S., Yuan, L., Wang, Y., 2013. A R2R3-MYB transcription factor from *Epimedium sagittatum* regulates the flavonoid biosynthetic pathway. PLoS One 8, e70778.

Japan Horticultural Plant Standard Color Chart 1984. Japan Color Research Institute, Tokyo, Japan.

Kamiishi, Y., Otani, M., Takagi, H., Han, D.S., Mori, S., Tatsuzawa, F., Okuhara, H., Kobayashi, H., Nakano, M., 2012. Flower color alteration in the liliaceous ornamental *Tricyrtis* sp. by RNA interference-mediated suppression of the chalcone synthase gene. Mol. Breed. 30, 671–680.

Katsumoto, Y., Fukuchi-Mizutani, M., Fukui, Y., Brugliera, F., Holton, T.A., Karan, M., Nakamura, N., Yonekura-Sakakibara, K., Togami, J., Pigeaire, A., Tao, G.Q., Nehra, N.S., Lu, C.Y., Dyson, B.K., Tsuda, S., Ashikari, T., Kusumi, T., Mason, J.G., Tanaka, Y., 2007. Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. Plant Cell Physiol. 48, 1589–1600.

Koes, R., Verweij, W., Quattrocchio, F., 2005. Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends Plant Sci. 10, 236–242.

Kortstee, A.J., Khan, S.A., Helderman, C., Trindade, L.M., Wu, Y., Visser, R.G., Brendolise, C., Allan, A., Schouten, H.J., Jacobsen, E., 2011. Anthocyanin production as a potential visual selection marker during plant transformation. Transgenic Res. 20, 1253–1264.

Kuwayama, S., Nakamura, T., Mizuta, Y., Oomiya, T., Nakano, M., 2005. Cross-compatibility in interspecific and intergeneric hybridization among the Colchicaceous ornamentals, *Gloriosa* spp., *Littonia modesta* and *Sandersonia aurantiaca*. Acta Hortic. 673, 421–427.

Laitinen, R.A.E., Ainasoja, M., Broholm, S.K., Teeri, T.H., Elomaa, P., 2008. Identification of target genes for a MYB-type anthocyanin regulator in Gerbera hybrida. J. Exp. Bot. 59, 3691–3703.

Lim, T.K., 2014. Pelargonium crispum. Edible Medicinal and Non Medicinal Plants. Springer, Dordrecht, pp. 72–76.

Matzke, A.J., Matzke, M.A., 1998. Position effects and epigenetic silencing of plant transgenes. Curr. Opin. Plant Biol. 1, 142–148.

Matzke, M.A., Primig, M., Trnovsky, J., Matzke, A.J.M., 1989. Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. EMBO J. 8, 643–649.

Mooney, M., Desnos, T., Harrison, K., Jones, J., Carpenter, R., Coen, E., 1995. Altered regulation of tomato and tobacco pigmentation genes caused by the delila gene of Antirrhinum. Plant J. 7, 333–339.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15, 473–497.

Naing, A.H., Lim, K.B., Kim, C.K., 2015. The usage of snapdragon *Delila* (*Del*) gene as a visible selection marker for the antibiotic-free transformation system. J. Plant Biol. 58, 110–116.

Nakamura, N., Fukuchi-Mizutani, M., Fukui, Y., Ishiguro, K., Suzuki, K., Tanaka, Y., 2010. Generation of pink flower varieties from blue *Torenia hybrida* by redirection of the flavonoid biosynthetic pathway from delphinidin to pelargonidin. Plant Biotechnol. 27, 375–383.

Nakatsuka, T., Nishihara, M., Mishiba, K., Yamamura, S., 2005. Temporal expression of flavonoid biosynthesis-related genes regulates flower pigmentation in gentian plants. Plant Sci. 168, 1309–1318.

Nakatsuka, T., Mishiba, K., Kubota, A., Abe, Y., Yamamura, S., Nakamura, N., Tanaka, Y., Nishihara, M., 2010. Genetic engineering of novel flower colour by suppression of anthocyanin modification genes in gentian. J. Plant Physiol. 167, 231–237.

Napoli, C., Lemieux, C., Jorgensen, R., 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans*. Plant Cell 2, 279–289.

Noda, N., Aida, R., Kishimoto, S., Ishiguro, K., Fukuchi-Mizutani, M., Tanaka, Y., Ohmiya, A., 2013. Genetic engineering of novel bluer-colored chrysanthemums produced by accumulation of delphinidin-based anthocyanins. Plant Cell Physiol. 54, 1684–1695.

Otani, M., Sharifi, A., Kubota, S., Oizumi, K., Uetake, F., Hirai, M., Hoshino, Y., Kanno, A., Nakano, M., 2016. Suppression of B function strongly supports the modified ABCE model in *Tricyrtis* sp. (Liliaceae). Sci. Rep. 6, 24549.

Otani, M., Kanemaki, Y., Oba, F., Shibuya, M., Funayama, Y., Nakano, M., 2018. Comprehensive isolation and expression analysis of the flavonoid biosynthesis-related genes in *Tricyrtis* sp. Biol. Plant. http://dx.doi.org/10.1007/s10535-018-0802-7. (in press).

Oud, J.S.N., Schneiders, H., Kool, A.J., van Grinsven, M.Q.J.M., 1995. Breeding of transgenic orange *Petunia hybrida* varieties. Euphytica 85, 403–409.

Peel, G.J., Pang, Y., Modolo, L.V., Dixon, R.A., 2009. The LAP1 MYB transcription factor orchestrates anthocyanidin biosynthesis and glycosylation in Medicago. Plant J. 59, 136–149.

Rabino, I., Mancinelli, A.L., 1986. Light, temperature, and anthocyanin production. Plant Physiol. 81, 922–924.

Ririe, K.M., Rasmussen, R.P., Wittwer, C.T., 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal. Biochem. 245, 154–160.

Saika, H., Sakamoto, W., Maekawa, M., Toki, S., 2011. Highly efficient visual selection of transgenic rice plants using green fluorescent protein or anthocyanin synthetic genes. Plant Biotechnol. 28, 107–110.

Singh, P., Khan, S., Kumar, S., Rahman, L., 2017. Establishment of an efficient *Agrobacterium*-mediated genetic transformation system in *Pelargonium graveolens*: an important aromatic plant. Plant Cell Tissue Organ Cult. 129, 35.

Terada, R., Shimamoto, K., 1990. Expression of CaMV35S-GUS gene in transgenic rice plants. Mol. Gen. Genet. 220, 389–392.

Wang, Y., Chen, B., Hu, Y., Li, J., Lin, Z., 2005. Inducible excision of selectable marker gene from transgenic plants by the cre/*lox* site-specific recombination system. Transgenic Res. 14, 605–614.

Yang, N.S., Christou, P., 1990. Cell type specific expression of a CaMV 35S-GUS gene in transgenic soybean plants. Dev. Genet. 11, 289–293.

Zhao, J., Li, Z.T., Chen, J., Henny, R.J., Gray, D.J., Chen, J., 2013. Purple-leaved *Ficus lyrata* plants produced by overexpressing a grapevine *VvMybA1* gene. Plant Cell Rep. 32, 1783–1793.

Zvi, M.M., Shklarman, E., Masci, T., Kalev, H., Debener, T., Shafir, S., Ovadis, M., Vainstein, A., 2012. *PAP1* transcription factor enhances production of phenylpropanoid and terpenoid scent compounds in rose flowers. New Phytol. 195, 335–345.