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Comprehensive isolation and expression analysis of the flavonoid biosynthesis-related genes in *Tricyrtis* spp.

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Abstract

Tricyrtis spp., which belong to the family *Liliaceae*, produce unique flowers, whose tepals have many reddish-purple spots. Although elucidation of a molecular mechanism of tepal spot formation and molecular breeding for flower colour alteration are desired for *Tricyrtis* spp., only one flavonoid biosynthesis-related gene, *TrCHS* encoding chalcone synthase (CHS), has been isolated so far. In the present study, comprehensive isolation and expression analysis of the other flavonoid biosynthesis-related genes were carried out in *Tricyrtis* sp. Six genes (*TrCHI*, *TrF3H*, *TrF3'H*, *TrFLS*, *TrDFR*, and *TrANS*) encoding biosynthetic enzymes chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H); flavonoid 3'-hydroxylase (F3'H), flavonol synthase (FLS), dihydroflavonol 4-reductase (DFR), and anthocyanin synthase (ANS) as well as three genes (*TrMYB1*, *TrbHLH2* and *TrWDR*) encoding transcription factors myeloblastosis 1 (MYB1), basic helix-loop-helix (bHLH), and WD40 repeats (WDRs) were newly isolated. Phylogenetic analysis showed that each isolated gene was classified into the monocotyledonous clade. Deduced amino acid sequences of DFRs showed that TrDFR has no substrate specificity. "Early" genes in the flavonoid biosynthetic pathway (*TrCHS*, *TrCHI*, and *TrF3H*) were constantly expressed in tepals during flower development, whereas expressions of "late" genes (*TrF3'H*, *TrFLS*, *TrDFR*, and *TrANS*) varied with the flower developmental stage. Expression patterns of the late genes were mostly correlated with those of transcriptional factor genes, indicating that the late genes may be under the control of a transcription factor complex consisted of TrMYB1, TrbHLH2, and TrWDR. Accumulation of anthocyanins in tepals occurred slightly after transcriptional upregulation of the late genes. Results obtained in the present study may be valuable for further studies on flower colour and flower colour pattern in *Tricyrtis* spp.

Additional key words: anthocyanins, flavonoids, flower colours, flower development, transcription factors.

Introduction

Flower colour and flower colour patterns are extremely important traits for ornamental plants. Anthocyanins, which are members of flavonoids, are found in various plant species and they are responsible for purple, blue, orange, and red pigmentation. Moreover, various flower colour patterns, such as spots, variegation, stripes, picotee, and gradation, are caused by a different pattern of anthocyanins accumulation. The molecular mechanism of anthocyanin accumulation through the flavonoid biosynthetic pathway is well understood (Fig. 1; Winkel-Shirley 2001). In higher plants, eight enzymes, chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), flavonoid 3',5'-hydroxylase (F3'5'H), flavonol synthase (FLS), dihydroflavonol 4-reductase (DFR), and anthocyanin synthase (ANS) are involved in the flavonoid biosynthesis from *p*-coumaroyl-CoA and malonyl-CoA to form anthocyanidins (pelargonidin, cyanidin, and delphinidin) and flavonols (kaempferol, quercetin, and myricetin). The structural genes encoding these enzymes have been comprehensively isolated from various plant species (Holton and Cornish 1995, Jaakola *et al*. 2002,

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Abbreviations: AN1- anthocyanin1; AN2 - anthocyanin2; ANS - anthocyanin synthase; bHLH - basic helix-loop-helix; CHI - chalcone isomerase; CHS - chalcone synthase; DFR - dihydroflavonol 4-reductase; FLS - flavonol synthase; F3H - flavanone-3-hydroxylase; F3'H - flavonoid 3'-hydroxylase; F3'5'H - flavonoid 3',5'-hydroxylase; HPLC - high performance liquid chromatography; MBW - MYB-bHLH-WDR; MYB - myeloblastosis; RACE - rapid amplification of cDNA ends; RT-PCR - reverse transcription-polymerase chain reaction; TF - transcription factor; TTG1 - transparent testa glabra 1; WDRs - WD40 repeats.
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Nakatsuka *et al*. 2005, 2008a, Chen *et al*. 2012, Guo *et al*. 2014, Li *et al*. 2014, Zhao *et al*. 2015a,b, Wang *et al*. 2018). Major anthocyanidins vary according to the plant species due to a lack of some flavonoid biosynthetic enzyme genes and/or substrate specificities of enzymes. For example, in *Rosa hybrida* (Katsumoto *et al*. 2007) and *Chrysanthemum morifolium* (Noda *et al*. 2013), no delphinidin-based anthocyanins are produced due to the absence of functional *F3'5'H* gene in their genome, and heterologous expression of *F3'5'H* genes from other plant species has induced accumulation of delphinidin-based anthocyanins. On the other hand, DFRs are responsible for the accumulation of limited types of anthocyanins. In *Petunia hybrida* (Gerats *et al*. 1982, Forkmann and Ruhnau 1987, Johnson *et al*. 2001) and *Cymbidium hybrida* (Johnson *et al*. 1999), DFRs cannot utilize dihydrokaempferol due to their substrate specificities, thus these species lack pelargonidin-based anthocyanins.

Expression of the flavonoid biosynthetic enzyme genes are regulated by a transcription factor (TF) complex consisted of R2R3-type myeloblastosis (R2R3-MYB), basic helix-loop-helix (bHLH), and WD40 repeats (WDRs) proteins (MBW complex) (Koes *et al*. 2005). R2R3-MYB TFs involved in the flavonoid biosynthesis can be classified into two subgroups, anthocyanin2 (AN2, such as *PhAN2* of *P. hybrida* and *AtPAP1* of *Arabidopsis thaliana*) and colorless1 (C1, such as *ZmC1* and *ZmPl* of *Zea mays*), according to their sequence homologs (Kranz *et al*. 1998, Jiang *et al*. 2004, Stracke *et al*. 2007). In dicotyledonous species, R2R3-MYBs belonging to the AN2 subgroup mainly regulate late genes in the flavonoid biosynthetic pathway (*FLS*, *F3'H*, *F3'5'H*, *DFR*, and *ANS*), whereas R2R3-MYBs of the C1 subgroup can regulate all flavonoid biosynthetic genes including early genes (*CHS*, *CHI*, and *F3H*) and late genes (Cone *et al*. 1993, Quattrocchio *et al*. 1999, Schwinn *et al*. 2006, Nakatsuka *et al*. 2008b, Chiu *et al*. 2010, Fornalé *et al*. 2010, Petroni and Tonelli 2011, Liu *et al*. 2016, Kunihiro *et al*. 2017). In monocotyledonous plants, most R2R3-MYB genes, such as *OgMYB1* of *Oncidium* sp. (Chiou and Yeh 2008), and *ZmC1* and *ZmPl* of *Z. mays* (Paz-Ares *et al*. 1987, Cone *et al*. 1993), belong to the C1 subgroup, and only *LhMYB6* and *LhMYB12* of *Lilium* sp. (Yamagishi *et al*. 2010), *AcMYB1* of *Allium cepa* (Schwinn *et al*. 2016), and *MaAN2* of *Muscari armeniacum* (Chen *et al*. 2017) are classified into the AN2 subgroup. Transcriptional regulation of the flavonoid biosynthetic enzyme genes in monocotyledonous species has not been well understood yet.

Tricyrtis spp., liliaceous ornamental plants, are cultivated for pot and garden uses. Most species and cultivars produce unique flowers, whose tepals have many reddish-purple spots of various sizes on a white to pale purple background. We have previously developed an efficient *Agrobacterium*-mediated transformation system for *Tricyrtis* sp. (Adachi *et al*. 2005, Nakano *et al*. 2006, Mori *et al*. 2008). In addition, *Tricyrtis* sp. requires only one year from *in vitro* regeneration to flowering (Nakano *et al*. 2006). Therefore, this plant seems to be suitable as a model for studying a molecular mechanism of tepal spot formation and for molecular breeding for flower colour alteration in liliaceous ornamental plants. However, only one flavonoid biosynthesis-related gene, *TrCHS* encoding CHS, has so far been isolated from *Tricyrtis* sp. (Kamiishi *et al*. 2012). Therefore, in the present study, we isolated flavonoid biosynthesis-related genes other than *TrCHS*. Expression analyses of *TrCHS* and newly isolated genes were also examined.

Materials and methods

Plant materials: Potted plants of *Tricyrtis* sp. cv. Shinonome were purchased from a local market and cultivated in a greenhouse without heating. Tepals of this cultivar have many reddish-purple spots on a pale purple background. Outer and inner tepals were collected from flowers at six different stages: S1 - flower bud of 6 to 10 mm in length, S2 - flower bud of 11 to 15 mm in length, S3 - flower bud of 15 to 20 mm in length, S4 - flower bud just before anthesis (over 21 mm in length), S5 - flower just after anthesis, and S6 - flower 3 d after anthesis (Fig. 4*A*).

Isolation of the flavonoid biosynthesis-related genes: Total RNA was extracted from tepals of young flower buds (S2 - S3) with *TRIzol* reagent (*Life Technologies*, Carlsbad, CA, USA) and treated with DNase I (*Life Technologies*) according to the manufacturer's instructions. To amplify full-length cDNA fragments, 3'-rapid amplification of cDNA ends (3'RACE) and 5'RACE was performed using the 3'-full RACE core set and the 5'-full RACE core set (*Takara Bio*, Shiga, Japan), respectively. Primers used for isolation of cDNA clones are listed in Table 1 Suppl. DNA sequencing was performed using the *ABI BigDye™ Terminator v. 3.1* cycle sequencing kits (*Applied Biosystems,* Foster City, CA, USA) with an automated sequencer (*ABI 3730xl Analyzer*; Applied Biosystems) according to the manufacturer's protocol.

Phylogenetic analysis: Phylogenetic trees were constructed using *MEGA 7.0* software with the neighbour-joining method based on *ClustalW* multiple alignments (Saitou and Nei 1987, Kumar *et al*. 2016). Accession numbers of genes used for the phylogenetic analysis are listed in Table 2 Suppl. The reliability of the trees was evaluated using the bootstrap resampling method (Felsenstein 1985) with 1 000 replicates.

Measurement of total anthocyanins: Total anthocyanins were extracted from 300 mg of outer and inner tepals of each flower developmental stage with the methanol-HCl method according to Rabino and Mancinelli (1986). Absorption (A) of the extracts at 530 and 657 nm was measured using a spectrophotometer (*Ultorospec 3000*; *GE Healthcare*, Little Chalfont, UK). Relative amount of total anthocyanins was calculated as $A_{530} - 0.25 \times A_{657}$. Each measurement was performed in triplicate.

High performance liquid chromatography (HPLC) analysis of anthocyanidins: Outer tepals were harvested from S5 flowers and stored at -80 °C until use. Anthocyanin extraction from tepals, hydrolyzation of crude extracts, and HPLC analysis were performed as described by Mori *et al*. (2002) and Kamiishi *et al*. (2012).

Semi-quantitative reverse transcription-polymerase chain reaction (RT-sqPCR): Total RNA was extracted from outer and inner tepals of each flower developmental stage, and leaves and stems of plants at the flowering stage. The total RNA (500 ng) was reverse-transcribed in a total volume of 0.02 cm^3 using the *PrimeScript*™ RT reagent kit (*Takara*, Shiga, Japan) according to the manufacturer's protocol. RT-sqPCR analysis was performed using the *EmeraldAmp*® *MAX PCR Master Mix* (*Takara*) on the *T100*™ thermal cycler (*Bio-Rad*, Hercules, CA, USA). Primer sets used are listed in Table 3 Suppl. Each PCR was performed in triplicate under the following conditions: initial denaturation at 94 °C for 1 min; 30 or 32 cycles at 95 °C for 30 s, at 60 °C for 30 s, and at 72 °C for 30 s. Amplified products were analysed by electrophoresis on 1.5 % (m/v) agarose gels. The actin gene of *Tricyrtis* sp. (*TrAct2*; AB196260 from the GenBank/EMBL/DDBJ databases) was used as an internal control.

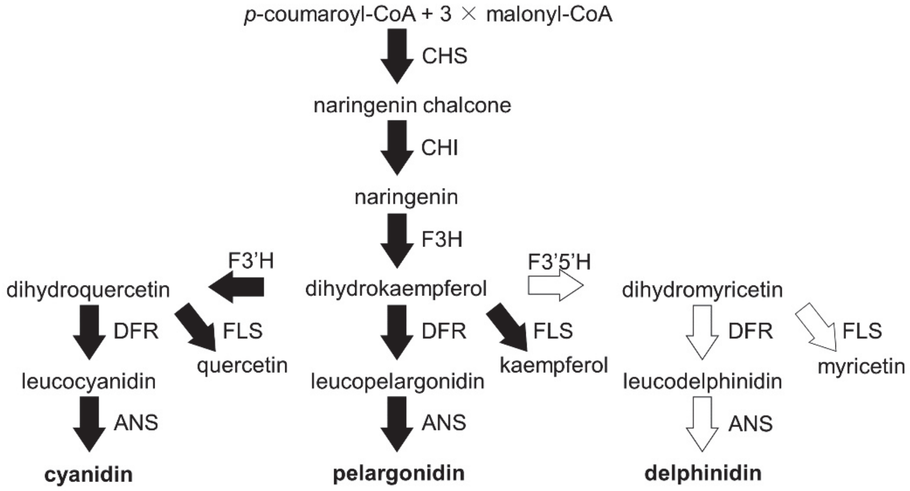


Fig. 1. Flavonoid biosynthetic pathway in plants. ANS - anthocyanin synthase, CHI - chalcone isomerase, CHS - chalcone synthase, DFR - dihydroflavonol 4-reductase, FLS - flavonol synthase, F3H - flavanone 3-hydroxylase, F3'H - flavonoid 3'-hydroxylase, F3'5'H - flavonoid 3',5'-hydroxylase. Probably non-existing pathway in *Tricyrtis* spp. is indicated by *white arrows*.

Results

Six genes encoding biosynthetic enzymes [*TrCHI* (accession number AB908277 in the GenBank/ EMBL/DDBJ databases), *TrF3H* (LC209222), *TrF3'H* (AB480691), *TrFLS* (LC103181), *TrDFR* (AB830112) and *TrANS* (LC209106)] and three genes encoding TFs [*TrMYB1* (AB856412), *TrbHLH2* (LC223741), and *TrWDR* (LC223742)] were isolated from *Tricyrtis* sp. The deduced amino acid sequences of these genes showed high homologies with those of corresponding genes of other plant species (Table 2 Suppl.). Fig. 2 and 1 Suppl. show phylogenetic trees based on the deduced amino acid sequence of each flavonoid biosynthesis-related gene. All the isolated genes from *Tricyrtis* sp. were classified into the monocotyledon clade, and most of these genes clustered closely with corresponding homologous genes of *Lilium*. The genes *TrMYB1* and *TrbHLH2* belong to the AN2 and AN1 subgroups, respectively (Figs. 2*A*,*B*). Fig. 3 shows an alignment of the deduced amino acid sequences of TrDFR and DFR homologous genes from other plant species. The N-terminal region of TrDFR contains a putative NADP-binding domain and a presumed substrate-binding region which is composed of 26 amino acid residues (Johnson *et al*. 2001).

Relative amount of total anthocyanins in outer and inner tepals of *Tricyrtis* sp. was measured at six flower developmental stages (Fig. 4*A*). Anthocyanins were

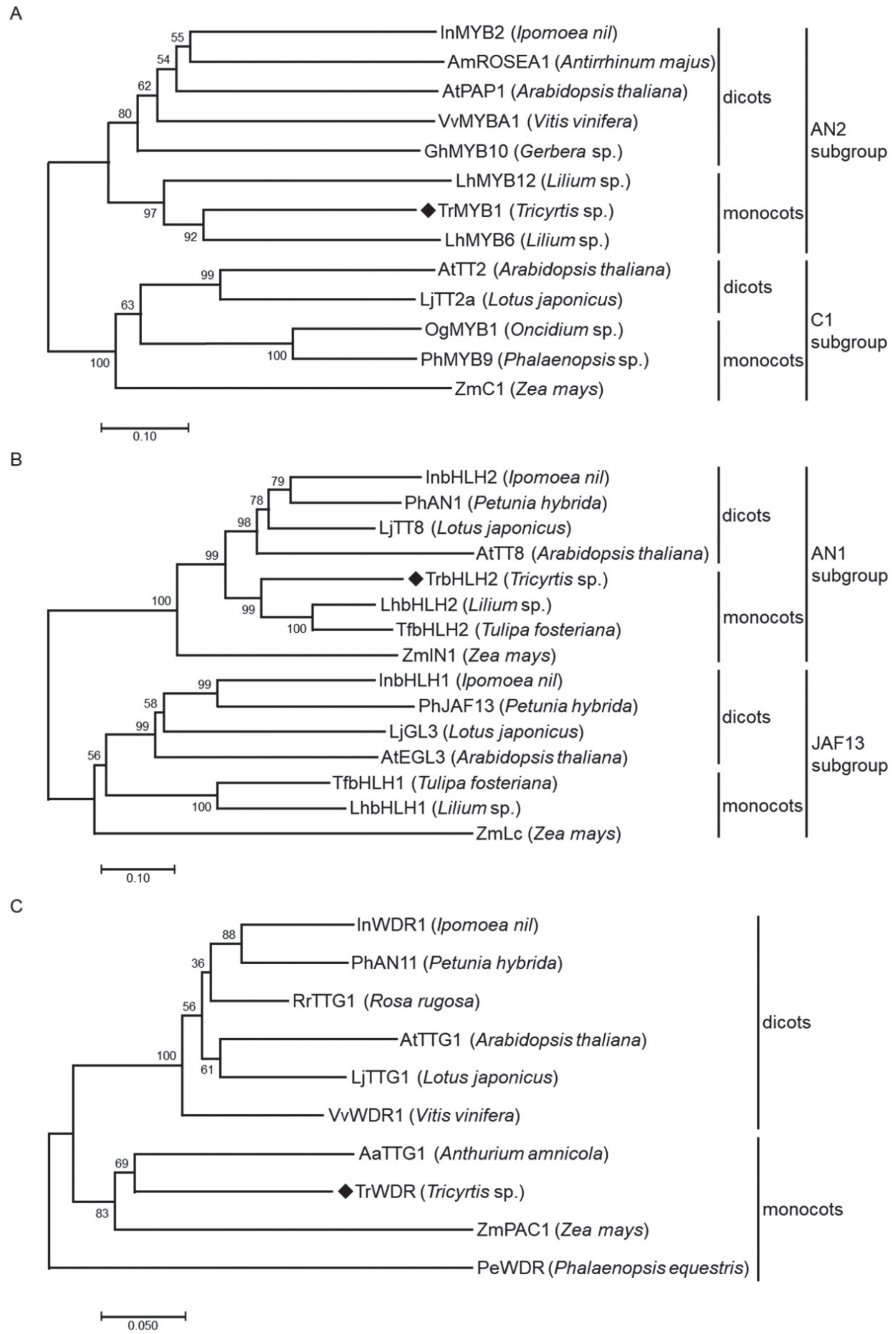
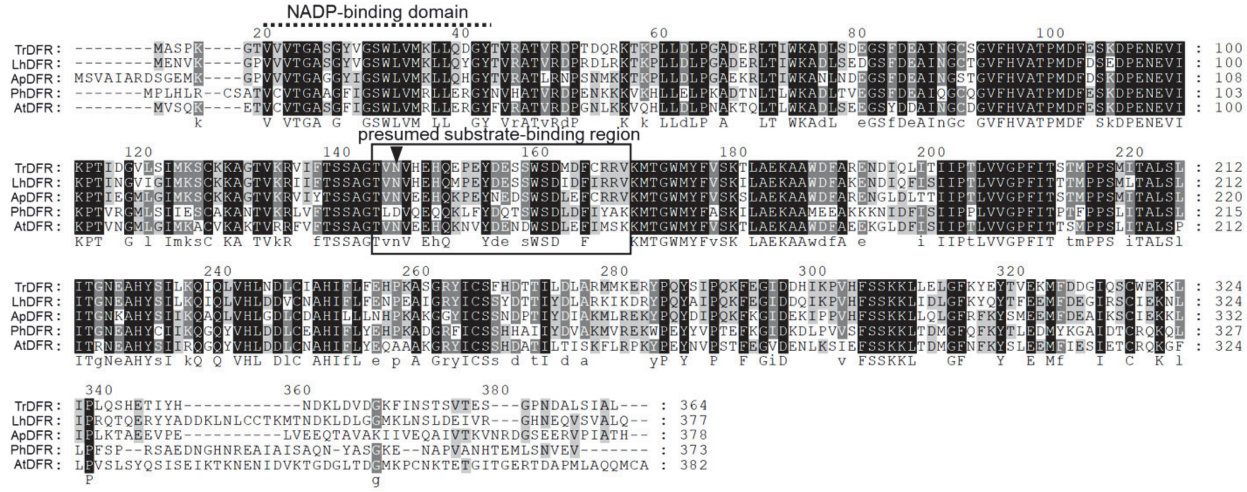


Fig. 2. Phylogenetic trees of deduced amino acid sequences of R2R3-MYB (*A*), bHLH (*B*), and WDR (*C*) homologous genes. Phylogenetic trees were constructed using *MEGA 7.0* software (neighbour-joining method based on *ClustalW* multiple alignments; 1 000 replicates). *Numerals* next to the branches indicate bootstrap values. *Scale bars* indicate 0.1 or 0.05 amino acid substitutions per site. Genes used for the phylogenetic analysis are listed in Table 2 Suppl.

detected at all stages, and the maximum accumulation was observed at S3 in both outer and inner tepals (Fig. 4*B*). HPLC analysis of anthocyanidins showed that tepals of *Tricyrtis* sp. mainly contained cyanidin and cyanidin 8-glucoside (Fig. 2 Suppl.). Other anthocyanidins such as pelargonidin and delphinidin were not detected.

Expression patterns of ten flavonoid biosynthesis-related genes, *TrCHS* (accession number AB478624 in the GenBank/EMBL/DDBJ databases), *TrCHI*, *TrF3H*, *TrF3'H*, *TrFLS*, *TrDFR*, *TrANS*, *TrMYB1*, *TrbHLH2*, and *TrWDR*, in outer and inner tepals at six flower developmental stages, leaves and stems of plants at the flowering stage were investigated by RT-sqPCR analysis (Fig. 5). For genes encoding flavonoid biosynthetic enzymes, early genes in the pathway (*TrCHS*, *TrCHI*, and *TrF3H*) were constantly expressed in both outer and inner



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                          20                  40                  60                  80                  100
TrDFR : --------MASPK----GTVVVTGASGYVGSWLVMKLLQDGYTVRATVRDPTDQRKTKPLLDLPGADERLTIWKADLSDEGSFDEAINGCSGVFHVATPMDFESKDPENEVI : 100
LhDFR : --------MENVK----GPVVVTGASGYVGSWLVMKLLQYGYTVRATVRDPRDLRKTKPLLDLPGADERLTIWKADLSEDGSFDEAINGCTGVFHVATPMDFDSEDPENEVI : 100
ApDFR : MSVAIARDSGEMK----GPVVVTGAGGYIGSWLVMKLLQHGYTVRATLRNPSNMKKTKPLLDLPGAEKRLTIWKANLNDEGSFDEAINGSTGVFHVATPMDFDSKDPENEVI : 108
PhDFR : -------MPLHLR--CSATVCVTGAAGFIGSWLVMRLLERGYNVHATVRDPENKKKVKHLLELPKADTNLTLWKADLTVEGSFDEAIQGCQGVFHVATPMDFESKDPENEVI : 103
AtDFR : --------MVSQK----ETVCVTGASGFIGSWLVMRLLERGYFVRATVRDPGNLKKVQHLLDLPNAKTQLTLWKADLSEEGSYDDAINGCDGVFHVATPMDFESKDPENEVI : 100
                    k      V VTGA G  GSWLVM LL  GY VrATvRdP    K k LLdLP A   LT WKAdL  eGSfDeAInGc GVFHVATPMDF SkDPENEVI

              120                 140                 160                 180                 200                 220
TrDFR : KPTIDGVLSIMKSCKKAGTVKRVIFTSSAGTVNVHEHQEPEYDESSWSDMDFCRRVKMTGWMYFVSKTLAEKAAWDFARENDIQLITIIPTLVVGPFITSTMPPSMITALSL : 212
LhDFR : KPTINGVIGIMKSCKKAGTVKRIIFTSSAGTVNVHEHQMPEYDESSWSDIDFIRRVKMTGWMYFVSKILAEKAAWDFAKENDIQFISIIPTLVVGPFITTTMPPSMLTALSL : 212
ApDFR : KPTIEGMLGIMKSCKKAGTVKRVIYTSSAGTVNVEEHQKPEYNEDSWSDLEFCRRVKMTGWMYFVSKSLAEKAAWDFARENGLDLTTIIPTLVVGPFITSTMPPSMITALSL : 220
PhDFR : KPTVRGMLSIIESCAKANTVKRLVFTSSAGTLDVQEQQKLFYDQTSWSDLDFIYAKKMTGWMYFASKILAEKAAMEEAKKKNIDFISIIPPLVVGPFITPTFPPSLITALSL : 215
AtDFR : KPTVNGMLGIMKACVKAKTVRRFVFTSSAGTVNVEEHQKNVYDENDWSDLEFIMSKKMTGWMYFVSKTLAEKAAWDFAEEKGLDFISIIPTLVVGPFITTSMPPSLITALSP : 212
        KPT  G l ImksC KA TVkR  fTSSAGTvnV EhQ   Yde sWSD  F    KMTGWMYFvSK LAEKAAwdfA e     i IIPtLVVGPFIT tmPPS iTALSl

                      240                 260                 280                 300                 320
TrDFR : ITGNEAHYSILKQIQLVHLNDLCIAHIFLFEHPKASGRYICSFHDTTILDLARMMKERYPQYSIPQKFEGIDDHIKPVHFSSKKLLELGFKYEYTVEKMFDDGIQSCWEKKL : 324
LhDFR : ITGNEAHYSILKQIQLVHLDDVCNAHIFLFENPEAIGRYICSSYDTTIYDLARKIKDRYPQYAIPQKFEGIDDQIKPVHFSSKKLIDLGFKYQYTFEEMFDEGIRSCIEKNL : 324
ApDFR : ITGNKAHYSIIKQAQLVHLGDLCDAHILLLNHPKAKGGYICSSNDPTIYDIAKMLREKYPQYDIPQKFKGIDEKIPPVHFSSKKLLQLGFRFKYSMEEMFDEAIKSCIEKKL : 332
PhDFR : ITGNEAHYCIIKQGQYVHLDDLCEAHIFLYEHPKADGRFICSSHHAIIYDVAKMVREKWPEYYVPTEFKGIDKDLPVVSFSSKKLTDMGFQFKYTLEDMYKGAIDTCRQKQL : 327
AtDFR : ITRNEAHYSIIRQGQYVHLDDLCNAHIFLYEQAAAKGRYICSSHDATILTISKFLRPKYPEYNVPSTFEGVDENLKSIEFSSKKLTDMGFNFKYSLEEMFIESIETCRQKGF : 324
        ITgNeAHYsI kQ Q VHL DlC AHIfL e p A GryICSs d tI d a      yP Y  P  F GiD     v FSSKKL   GF   Y  E Mf   I  C  K l

          340                 360                 380
TrDFR : IPLQSHETIYH------------NDKLDVDGKFINSTSVTES---GPNDALSIAL--- : 364
LhDFR : IPRQTQERYYADDKLNLCCTKMTNDKLDLGGMKLNSLDEIVR---GHNEQVSVALQ-- : 377
ApDFR : IPLKTAEEVPE----------LVEEQTAVAKIIVEQAIVTKVNRDGSEERVPIATH-- : 378
PhDFR : LPFSP--RSAEDNGHNREAIAISAQN-YASGKE--NAPVANHTEMLSNVEV------- : 373
AtDFR : LPVSLSYQSISEIKTKNENIDVKTGDGLTDGMKPCNKTETGITGERTDAPMLAQQMCA : 382
         P                            g
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Fig. 3. Alignment of deduced amino acid sequences of *TrDFR* from *Tricyrtis* sp. and those of DFR homologous genes from other plant species. Identical amino acid residues are shown with *white characters on the black background*. *Black dotted line* indicates a putative NADP-binding domain. *Boxed region* indicates a presumed substrate-binding region composed of 26 amino acid residues, and substrate specificity is directly associated with a single amino acid residue represented by an *arrow head*. Genes used for the alignment are listed in Table 2 Suppl.

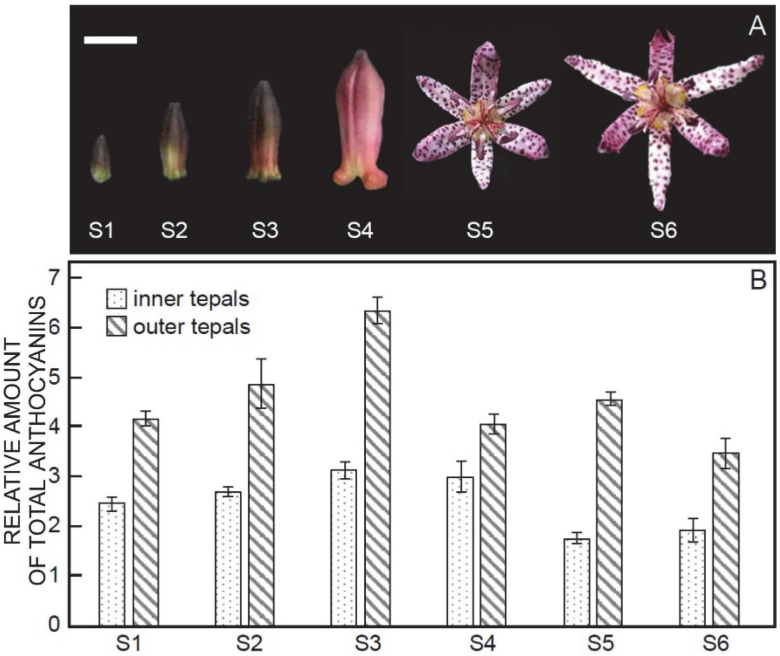

Fig. 4. Flower development and anthocyanin accumulation in tepals of *Tricyrtis* sp. *A* - Developmental stages of the flower; *bar* = 1 cm. *B* - Relative amount of anthocyanins (calculated as A_{530} - 0.25 × A_{657}) in tepals of each flower developmental stage. Means ± SEs, *n* = 3.

tepals, leaves, and stems. On the other hand, expressions of late genes in the biosynthetic pathway (*TrF3'H*, *TrFLS*, *TrDFR*, and *TrANS*) varied with the flower developmental stage. Late genes expressed strongly in both inner and outer tepals at S1 and S2. In outer tepals, expressions of late genes temporarily decreased at S3 and then increased again at S4. At S5 (just after anthesis), expressions of late genes decreased in both outer and inner tepals. Expressions of *TrF3'H* and *TrFLS* increased again at S6. The maximum expressions of genes encoding three transcriptional factors, *TrMYB1*, *TrbHLH2*, and *TrWDR*, were observed in both outer and inner tepals at S2.

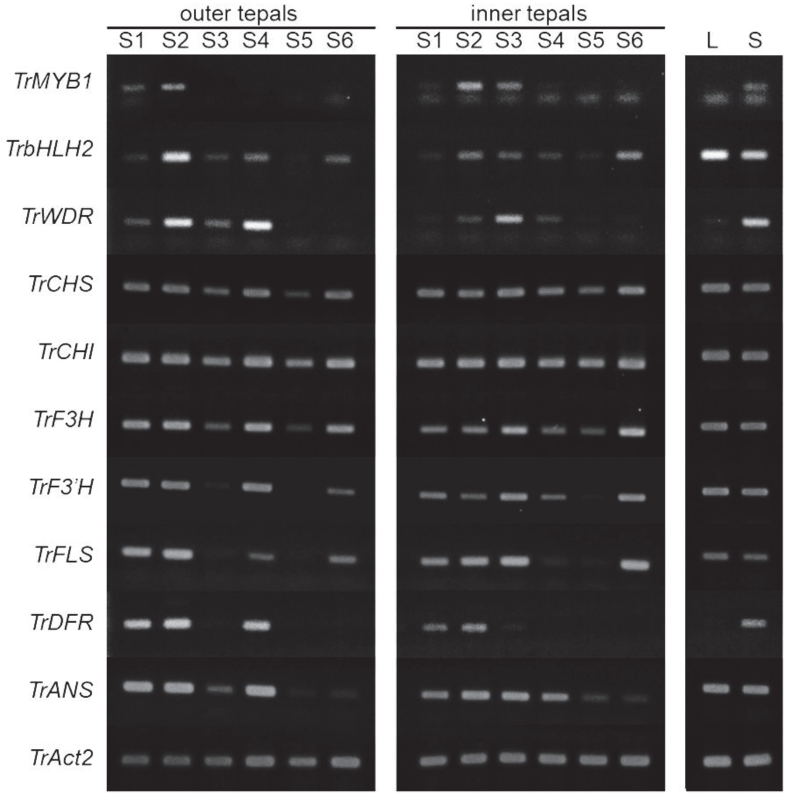

Fig. 5. Expression patterns of three flavonoid biosynthesis-related genes in outer and inner tepals, leaves (L), and stems (S) of *Tricyrtis* sp. at different developmental stages (RT-sqPCR analysis; *TrAct2* was used as internal control).

Discussion

In the present study, totally nine flavonoid biosynthesis-related genes, six genes encoding flavonoid biosynthetic enzymes (*TrCHI*, *TrF3H*, *TrF3'H*, *TrFLS*, *TrDFR*, and *TrANS*) and three genes encoding TFs (*TrMYB1*, *TrbHLH2,* and *TrWDR*), were newly isolated from *Tricyrtis* sp. cv. Shinonome. Phylogenetic and sequence homology analyses showed that each isolated gene has a close relationship with a corresponding gene of other monocotyledonous species.

In tepals, the only cyanidins were detected, but other anthocyanidins, such as pelargonidin and delphinidin, were not detected. Likewise, cyanidins have been reported to be mainly accumulated in tepals of some *Lilium* spp. (Nørbæk and Kondo 1999) and *Tricyrtis formosana* (Tatsuzawa *et al*. 2004). In addition, *F3'5'H* genes have never been isolated from any liliaceous species so far. Thus, liliaceous plants including *Tricyrtis* spp. might have no functional *F3'5'H* genes in the genome (Fig. 1).

Difference of a single amino acid residue in the substrate-binding region of DFR defines substrate specificity (Johnson *et al*. 2001). Generally, DFRs with asparagine residue (N) in that position can utilize all the three dihydroflavonols as substrates, whereas DFRs with aspartic acid (D) cannot catalyse dihydrokaempferol efficiently (Gerats *et al*. 1982, Forkmann and Ruhnau 1987, Johnson *et al*. 1999, 2001). Since TrDFR has an asparagine residue in that position, TrDFR may have no substrate specificity. Thus, the type of anthocyanidins in tepals of *Tricyrtis* sp. might be determined by the balance of gene expression, timing, and/or enzymatic activities of flavonoid biosynthetic enzymes such as TrF3'H and TrDFR (in addition to the absence of a functional *F3'5'H* gene).

Expressions of genes encoding flavonoid biosynthetic enzymes has been reported to be regulated by the MBW complex consisted of R2R3-MYB, bHLH, and WDR TFs (Koes *et al.* 2005). The bHLHs involved in the flavonoid biosynthesis can be classified into two subgroups, anthocyanin1 (AN1, such as *PhAN1* in *Petunia hybrida*) and JAF13 (such as *PhJAF13* in *P. hybrida*) according to their sequence homologies (Spelt *et al.* 2000). Montefiori *et al.* (2015) reported that bHLHs belonging to the AN1 subgroup can form the MBW complex, which directly regulates expression of the flavonoid biosynthetic enzyme genes. On the other hand, bHLHs belonging to the JAF13 subgroup indirectly regulate the flavonoid biosynthesis through transcriptional activation of *bHLH* genes of the AN1 subgroup. Phylogenetic analysis showed that TrbHLH2 was classified into the AN1 subgroup, indicating that TrbHLH2 may directly regulate expression of the flavonoid biosynthetic enzyme genes. The WDRs are involved in a wide variety of plant processes, including cell division, meristem organization, light signalling, floral development, secondary metabolism, and innate immunity (Smith *et al.* 1999, Van Nocker and Ludwig 2003, Perfus-Barbeoch *et al.* 2004). In *A. thaliana*, *TRANSPARENT TESTA GLABRA 1* (*AtTTG1*) is the only gene that encodes a WDR involved in the regulation of the flavonoid biosynthesis (Walker *et al.* 1999). The TrWDR showed a close relationship with AtTTG1, suggesting that TrWDR may act as a regulator of the flavonoid biosynthesis. Generally, the target gene specificity of the MBW complex is conferred by R2R3-MYBs (Hichri *et al.* 2011, Petroni and Tonelli 2011). TrMYB1 forms a cluster with R2R3-MYBs belonging to the AN2 subgroup that mainly regulate transcription of late genes in the flavonoid biosynthetic pathway (Cone *et al.* 1993, Quattrocchio *et al.* 1999, Schwinn *et al.* 2006, Chiou and Yeh 2008, Nakatsuka *et al.* 2008b, Chiu *et al.* 2010, Fornalé *et al.* 2010, Petroni and Tonelli 2011, Liu *et al.* 2016, Kunihiro *et al.* 2017). In tepals of *Tricyrtis* sp., early genes in the flavonoid biosynthetic pathway (*TrCHS*, *TrCHI*, and *TrF3H*) were constantly expressed during flower development, whereas expressions of late genes (*TrF3'H*, *TrFLS*, *TrDFR*, and *TrANS*) varied with the flower developmental stage. Moreover, expression patterns of the late genes were correlated with that of *TrMYB1*, except for outer tepals at S4. Combining the results from phylogenetic and expression analyses, the MBW complex consisted of TrMYB1, TrbHLH2, and TrWDR might regulate expression of the late genes in *Tricyrtis* sp. In outer tepals, the late genes were upregulated at S4 without increased expression of *TrMYB1*, suggesting that other *R2R3-MYB* genes might be present in the genome of *Tricyrtis* sp.

As described above, TrMYB1 belongs to the AN2 subgroup and may be involved in the regulation of the late genes. On the other hand, *LhMYB12* of *Lilium* sp. has been reported to regulate both early and late genes despite belonging to the AN2 subgroup (Lai *et al.* 2012, Suzuki *et al.* 2016). In order to clarify the detailed regulatory mechanisms of genes encoding flavonoid biosynthetic enzymes by R2R3-MYBs, it is necessary to carry out further investigation for R2R3-MYBs in various plant species, especially monocotyledonous ones.

In tepals of *Tricyrtis* sp., anthocyanins accumulation increased slightly after transcriptional upregulation of the late genes. In both outer and inner tepals, the maximum expressions of the flavonoid biosynthesis-related genes were observed at S2, whereas the maximum accumulation of anthocyanins was observed at S3. In outer tepals, expressions of the late genes were temporarily increased at S4, and relative amounts of total anthocyanins slightly increased at S5. Similar observations have been reported for *Viola* × *wittrockiana* (Li *et al.* 2014), *Rhododendron* × *pulchrum* (Nakatsuka *et al.* 2008a), *Lilium* spp. (Lai *et al.* 2012), and *Paeonia suffruticosa* (Zhao *et al.* 2015a). In both outer and inner tepals, *TrF3'H* and *TrFLS* strongly expressed at S6, suggesting that the flavone biosynthesis in tepals might occur mainly after anthesis.

In the present study, flavonoid biosynthesis-related genes were isolated and characterized in the liliaceous ornamental plant *Tricyrtis* sp. cv. Shinonome. Basal information for elucidation of the molecular mechanism of tepal spot formation and for molecular breeding for flower colour alteration has been obtained. We have previously produced transgenic *Tricyrtis* sp. plants with white flowers by RNA interference (RNAi)-mediated suppression of *TrCHS* (Kamiishi *et al.* 2012). Production of transgenic *Tricyrtis* spp. plants with novel flower colour phenotypes is now in progress by overexpression or suppression of genes isolated in the present study. Elucidation of the mechanism of tepal spot formation is also now in progress by investigating gene(s) showing tepal spot-specific expression.

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