

RESEARCH PAPER

# Isolation and characterization of GtMYBP3 and GtMYBP4, orthologues of R2R3-MYB transcription factors that regulate early flavonoid biosynthesis, in gentian flowers

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## Abstract

Flavonoids are one of the major plant pigments for flower colour. Not only coloured anthocyanins, but also co-pigment flavones or flavonols, accumulate in flowers. To study the regulation of early flavonoid biosynthesis, two R2R3-MYB transcription factors, GtMYBP3 and GtMYBP4, were identified from the petals of Japanese gentian (*Gentiana triflora*). Phylogenetic analysis showed that these two proteins belong to the subgroup 7 clade (flavonol-specific MYB), which includes *Arabidopsis* AtMYB12, grapevine VvMYB1, and tomato SIMYB12. GtMYBP3 and GtMYBP4 transcripts were detected specifically in young petals and correlated with the profiles of flavone accumulation. Transient expression assays showed that GtMYBP3 and GtMYBP4 enhanced the promoter activities of early biosynthetic genes, including flavone synthase II (*FNSII*) and flavonoid 3'-hydroxylase (*F3'H*), but not the late biosynthetic gene, flavonoid 3',5'-hydroxylase (*F3'5'H*). GtMYBP3 also enhanced the promoter activity of the chalcone synthase (*CHS*) gene. In transgenic *Arabidopsis*, overexpression of GtMYBP3 and GtMYBP4 activated the expression of endogenous flavonol biosynthesis genes and led to increased flavonol accumulation in seedlings. In transgenic tobacco petals, overexpression of GtMYBP3 and GtMYBP4 caused decreased anthocyanin levels, resulting in pale flower colours. GtMYBP4-expressing transgenic tobacco flowers also showed increased flavonols. As far as is known, this is the first functional characterization of R2R3-MYB transcription factors regulating early flavonoid biosynthesis in petals.

**Key words:** Early flavonoid biosynthesis, flavone, flower colour, Japanese gentian, R2R3-MYB, transcription factor.

## Introduction

Natural flower pigments generally consist of complex secondary metabolites, such as flavonoids, carotenoids, and betalains, depending on the plant species. Flavonoids are the most characterized secondary metabolites in higher plants and have various biological functions, such as flower pigmentation, pollen fertility, plant–microbe interaction, and protection from UV radiation (reviewed in Mol *et al.*, 1998). Determination of the flavonoid biosynthetic pathway is of great interest to plant scientists and many structural genes involved in flavonoid biosynthesis have been cloned and studied in maize kernels (*Zea mays*), petunia flowers (*Petunia hybrida*), snapdragon flowers (*Antirrhinum*

*majus*), and in *Arabidopsis* seed coats (*Arabidopsis thaliana*) (Fig. 1; Holton and Cornish, 1995; Mol *et al.*, 1998; Winkel, 2006).

Japanese endemic gentian plants (*Gentiana triflora*) have brilliant blue flowers and are popular floricultural plants in Japan (Nishihara *et al.*, 2008). The petals of Japanese gentian accumulate a polyacylated anthocyanin, termed gentiodelphin (delphinidin 3-*O*-glucoside-5,3'-*O*-caffeoylglucoside), and flavone derivatives (Goto *et al.*, 1982; Yoshida *et al.*, 2000; Nakatsuka *et al.*, 2005). The structural genes involved in the gentiodelphin and flavone biosynthetic pathways have been isolated

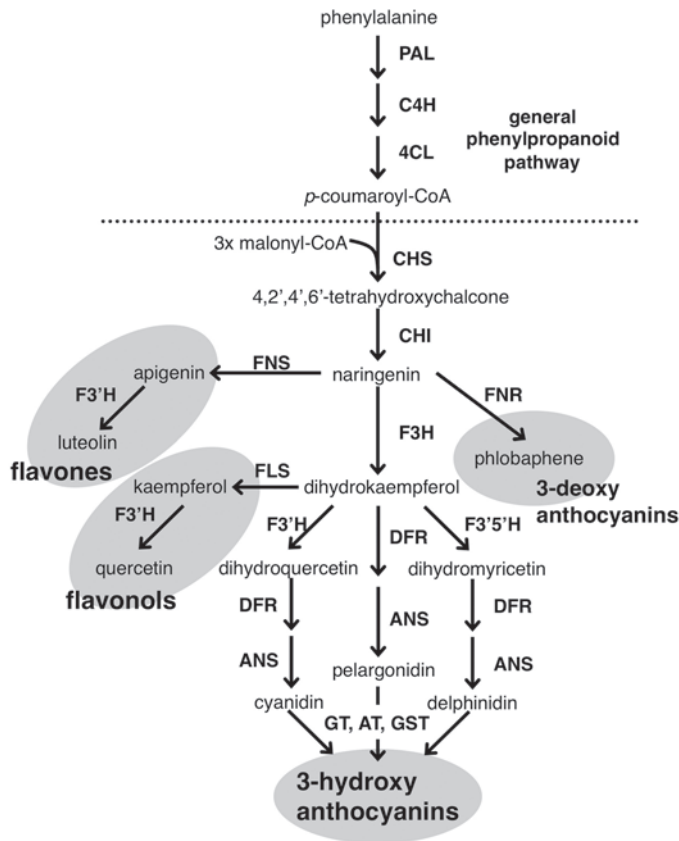
and characterized (Tanaka et al., 1996; Kobayashi et al., 1998; Nakatsuka et al., 2005, 2008a). Thus, the blue pigmentation and its development have been extensively studied in Japanese gentian (Yoshida et al., 2009).

In flavonoid biosynthesis, two clusters of co-regulated structural genes can generally be distinguished: early biosynthetic genes, which are involved in the synthesis of flavones, flavonols, and phlobaphenes; and late biosynthetic genes, which are involved in proanthocyanin and anthocyanin biosynthesis (Fig. 1; Mol et al., 1998; Quattrocchio et al., 2006; Gonzalez et al., 2008). Transcription factors for the anthocyanin and proanthocyanin biosynthetic pathways are mainly members of the R2R3-MYB, basic helix-loop-helix (bHLH), and WD40 repeats (WDR) protein families. Complexes of these transcription factors regulate the expression of structural genes in the late biosynthetic pathway (Broun, 2005; Koes et al., 2005). In maize, C1 (R2R3-MYB) requires interaction with R (bHLH) to activate anthocyanin biosynthesis (Lloyd et al., 1992). In petunia flowers, AN2 (R2R3-MYB) interacts with two distinct bHLH factors, JAF13 or AN1, both of which share a high sequence

similarity with the maize R and snapdragon DELILA proteins (Spelt et al., 2000). PAPI/PAP2 are also C1 homologues in *Arabidopsis* and are involved in proanthocyanin accumulation in seed coats (Borevitz et al., 2000). In gentian, GtMYB3 interacts with GtbHLH1, and the complex of these two proteins activates the expression of flavonoid 3',5'-hydroxylase (*F3'5'H*) and anthocyanin 5,3'-aromatic acyltransferase (*5,3'AT*) genes, which belong to the late flavonoid biosynthetic pathway (Nakatsuka et al., 2008b). However, the GtMYB3/GtbHLH1 complex could not activate the expression of the chalcone synthase (*CHS*) gene, which belongs to the early biosynthetic pathway. Thus, the activation of many R2R3-MYB proteins regulating flavonoid biosynthetic pathways depends on an interaction with bHLH proteins. R2R3-MYB proteins that interact with bHLH proteins share a common motif in the N-terminal R3 repeat (Grotewold et al., 2000). However, the transcription factors regulating early flavonoid biosynthesis in gentian flowers remain unknown.

Maize P1 (ZmP1) is an R2R3-MYB that is active without binding a bHLH protein. ZmP1 controls a subset of genes for 3-deoxyflavonoids and phlobaphene biosyntheses in kernel pericarp and cob tissue (Grotewold et al., 1994). A transient expression assay using a maize cell suspension demonstrated that P1 can activate the transcription of the flavanone reductase gene (*Al*, *FNR*) but cannot activate the flavonoid 3-*O*-glucosyltransferase gene (*Bz1*, *3GT*). Genetic analysis also showed that the *ZmP1* locus coincides with a major quantitative trait locus determining the levels of maysin, a C-glycosyl flavone (Byrne et al., 1996; Zhang et al., 2003; Cocciolone et al., 2005). In *Arabidopsis*, AtMYB12/PFG2, AtMYB11/PFG1, and AtMYB111/PFG3, which share amino acid sequence similarity with ZmP1, regulate individual flavonol accumulation in differential organs of developing seedlings (Mehrtens et al., 2005; Stracke et al., 2007). They activate the expression of *CHS*, chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), flavonol synthase (*FLS*), and flavonoid 3'-hydroxylase (*F3'H*), but do not influence the expression of dihydroflavonol 4-reductase (*DFR*). In grapevine, the expression of *VvMYBF1* correlated with flavonol accumulation and the expression of *VvFLS1* (Czemmel et al., 2009). In addition, deficiency of SIMYB12, the tomato orthologue of AtMYB12, resulted in pink fruit lacking the ripening-dependent accumulation of the yellow flavonoid naringenin chalcone in the fruit peel (Ballester et al., 2010). These R2R3-MYBs were classified into subgroup 7 (Dubos et al., 2010) and activated specific gene sets of the early flavonoid biosynthetic pathway (Stracke et al., 2007). However, in spite of the demonstrated accumulation of early flavonoid biosynthetic metabolites, such as flavonol and flavone, no transcription factors regulating the early steps of flavonoid biosynthesis have been identified in the petals of floral plants.

In the present study, two P1 orthologues, *GtMYBP3* and *GtMYBP4*, were isolated and characterized in Japanese gentian. The expression profiles of both genes correlated with flavone accumulation in petals and activated the expression of *CHS*, flavone synthase II (*FNSII*), and *F3'H*, belonging to early flavonoid biosynthetic pathway. Functional analyses of *GtMYBP3* and *GtMYBP4* were performed in transgenic *Arabidopsis* and tobacco plants. The results strongly suggested that both *GtMYBP3* and *GtMYBP4* regulate the early steps of the flavonoid biosynthetic



**Fig. 1.** Flavonoid biosynthetic pathway in higher plants. 4CL, 4-coumarate:CoA ligase; ANS, anthocyanin synthase; AT, anthocyanin acyltransferase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3'5'H, flavonoid 3',5'-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; FNR, flavanone reductase; FNS, flavone synthase; GST, glutathione S-transferase; GT, anthocyanin O-glucosyltransferase; PAL, phenylalanine ammonia lyase.

pathway. This is the first report to investigate the function of P1 orthologues in floral organs.

## Materials and methods

### Plant materials

Japanese gentian (*G. triflora* cv. Maciry) plants were grown in the fields of the Iwate Agricultural Research Center (Iwate prefecture, Japan). The developmental stages of petal samples were defined as described by Nakatsuka *et al.* (2005).

### Isolation of gentian R2R3-MYB transcription factors

Total RNA was isolated from petals at flower developmental stages 1–3. cDNA was synthesized using a Takara RNA PCR kit (AMV) version 3.0 (Takara-bio, Ohtsu, Japan). Degenerate primers were designed from the conserved DNA-binding domain of R2R3-MYBs controlling flavonoid biosynthesis from other plant species (Supplementary Table S1, available at JXB online). Other degenerate primers (pMybU and pMybL; Rabinowicz *et al.*, 1999) were also used. Reaction conditions consisted of pre-heating at 94 °C for 90 s; 40 cycles of 95 °C for 20 s, 50 °C for 40 s, and 72 °C for 1 min; and extension at 72 °C for 10 min. Amplified fragments of approximately 180 bp were subcloned into the pCR4 TA TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) and sequenced using a Big-Dye terminal cycle sequencing kit version 1.1 and an ABI PRISM 3130 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were conceptually translated into amino acid sequences using GENETYX-MAC version 12 (GENETYX, Tokyo, Japan) and compared using the BLAST network service from the National Center for Biotechnology Information (NCBI). A phylogenetic tree was constructed using MEGA version 4 (Tamura *et al.*, 2007).

To obtain full-length cDNAs of *GtMYBP3* and *GtMYBP4*, 3'- and 5'-rapid amplification of cDNA ends (RACE) was performed using a GeneRacer kit (Invitrogen). The amplified fragments were subcloned and sequenced as described above.

Northern blot analysis was performed in gentian using *GtMYBP3* and *GtMYBP4* probes (Supplementary Table S1), as described by Nakatsuka *et al.* (2005).

### Yeast two-hybrid analysis

The yeast two-hybrid assay was performed using the Matchmaker Yeast Two-Hybrid System 3 (Clontech, Mountain View, CA, USA) as described previously (Nakatsuka *et al.*, 2008b). Briefly, the open reading frame sequences of *GtMYBP3* and *GtMYBP4* were cloned into the pGAD-T7 and pGBK-T7 vectors. *GtMYB3* and *GtbHLH1* constructs (Nakatsuka *et al.*, 2008b) were also used.

### Isolation of 5'-UTRs of *GtFNSII* and *GtF3'H* in gentian

The 5'-untranscribed regions of gentian *GtFNSII* and *GtF3'H* were identified by inverse PCR, using primers shown in Supplementary Table S1 and as described by Nakatsuka *et al.* (2008b). Amplified fragments of about 1.8 kb for *GtFNSII* and 1.6 kb for *GtF3'H* were subcloned and sequenced as described above. Reporter vectors were constructed to contain the firefly luciferase (LUC) gene under the control of the gentian *GtFNSII* (accession number AB733018) or *GtF3'H* (AB733017) promoters. *GtCHSpro-LUC* and *GtF3'5'Hpro-LUC* constructs (Nakatsuka *et al.*, 2008b) were also used.

### Transient expression assay using *Arabidopsis* suspension cells

To evaluate whether *GtMYBP3* and *GtMYBP4* are responsible for the regulation of early flavonoid biosynthesis, transient expression assays were performed using protoplasts from *Arabidopsis* T87 suspension cells (Axelos *et al.*, 1992). The open reading frame sequences of *GtMYBP3* and *GtMYBP4* were inserted into the p35Spro expression vector under

the control of the CaMV35S promoter and NOS terminator, resulting in p35Spro-*GtMYBP3* and p35Spro-*GtMYBP4*, respectively. pBI221 (Clontech) was used as a negative control vector. p35Spro-RLUC, the *Renilla* luciferase (RLUC) gene under the control of the CaMV35S promoter, was used as a transformation control. Protoplast isolation and PEG-transfection experiments were performed as described by Hartmann *et al.* (1998). Firefly and *Renilla* luciferase activities were measured by the dual-Glo luciferase assay system (Promega, Madison, WI, USA) and Luminescencer JNR II (ATTO, Tokyo, Japan), according to the manufacturers' instructions. At least five independent transfections were done for each plasmid combination to demonstrate reproducibility.

### Vector construct and production of stable *Arabidopsis* and tobacco transformants

p35Spro-*GtMYBP3* and p35Spro-*GtMYBP4* was inserted into binary vectors, pSkan35SGUS (kanamycin resistance) and pSMABR35S-sGFP (Mishiba *et al.*, 2010), to produce pSkan-35Spro-*GtMYBP3* and pSMABR-35Spro-*GtMYBP4*, respectively. The constructs were then transformed into *Agrobacterium tumefaciens* EHA101. *A. thaliana* ecotype col-1 was transformed by floral dip method, as described by Clough and Bent (1998). Positive transformants were selected on germination medium supplemented with 50 mg l<sup>-1</sup> kanamycin or 6 mg l<sup>-1</sup> bialaphos, and then T<sub>2</sub> seeds were obtained following self-pollination.

Tobacco plants (*Nicotiana tabacum* cv. SR-1), aseptically grown from seeds for about 1 month, were transformed via an *A. tumefaciens*-mediated leaf disc procedure (Horsch *et al.*, 1985) and selected using 200 mg l<sup>-1</sup> kanamycin or 5 mg l<sup>-1</sup> bialaphos. After rooting and acclimatization, regenerated plants were grown in a greenhouse to set seeds by self-pollination. T<sub>1</sub> transgenic plant lines were used for further analyses.

### Flavonoid analysis in transgenic *Arabidopsis* and tobacco plants

The flavonol accumulation in T<sub>2</sub> transgenic *Arabidopsis* seedlings was visualized by diphenylboric acid 2-aminoethyl ester (DPBA), as described by Stracke *et al.* (2007). Five-day-old seedlings grown on germination medium with 3 mg l<sup>-1</sup> norflurazon were stained by 0.25% (w/v) DPBA. Fluorescence images were visualized under UV light on a stereoscopic microscope (Olympus, Tokyo, Japan). The amount of anthocyanin and flavonol pigments in petals of transgenic tobacco plants were measured as described by Nakatsuka *et al.* (2007).

### Quantitative real-time PCR in transgenic *Arabidopsis* and tobacco plants

Total RNA was isolated from the seedlings of transgenic *Arabidopsis* after germination for 7 days using an RNeasy Plant Mini kit (Qiagen, Venlo, The Netherlands). Total RNA of transgenic tobacco was also isolated from their petals at floral developmental stage 3 using a FastRNA Green kit (Q-Bio gene, Irvine, CA, USA).

Quantitative real-time PCR (qRT-PCR) was performed on a StepOne plus (Applied Biosystems) using SYBR GreenER qPCR Super Mix (Invitrogen). cDNA was synthesized from total RNA after removal of genomic DNA using PrimeScript RT reagent kit with gDNA eraser (Takara-bio), according to the manufacturer's instructions. Reaction mixtures (10 µl) included the following components: 1×master mix, 0.2 µM of each primer (Supplementary Table S2) and 1 µl cDNA. Cycle conditions were 95 °C for 20 s and then 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The expression levels of each gene were calibrated by the expression of actin (*AtACT*, *Arabidopsis*) or ubiquitin (*NtUBQ*, tobacco) genes.

## Results

### Cloning of P1 orthologues from gentian flowers

Degenerate PCR was used to isolate R2R3-MYB transcription factor genes from gentian flowers. More than 200 clones were

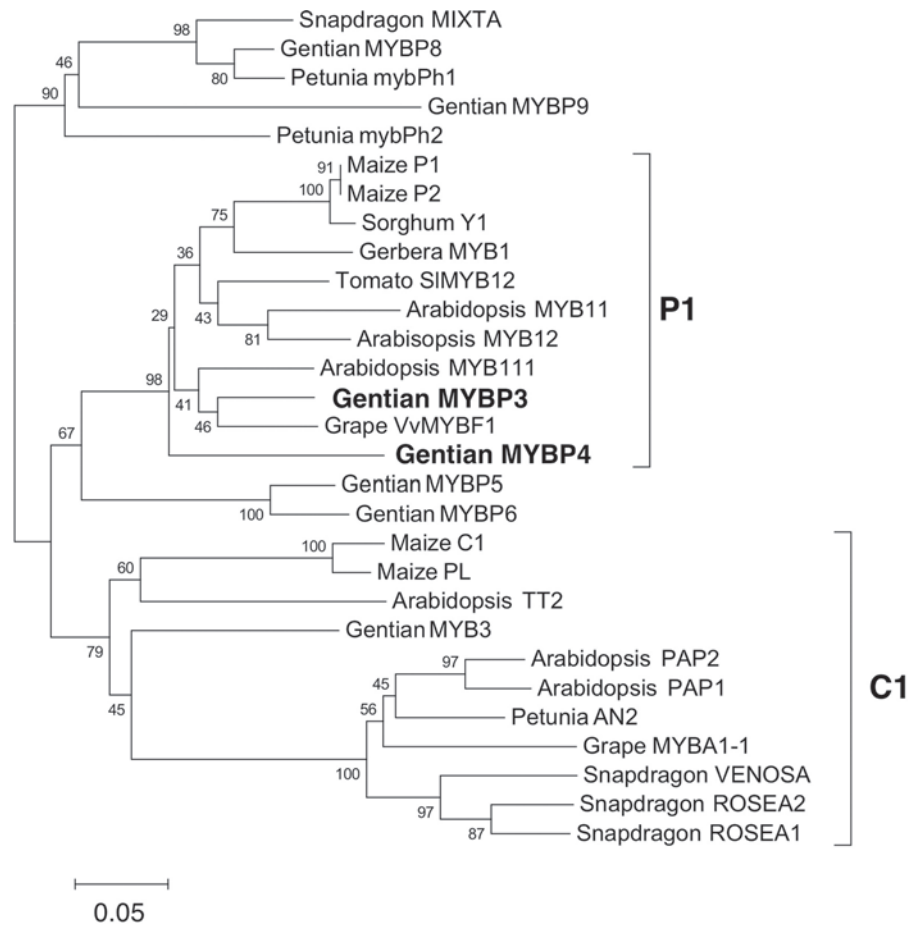
classified into 24 groups based on a similarity search. Among them, the deduced amino acid sequences of two clones exhibited high similarities with ZmP1 and AtMYB12. Full-length cDNAs for these two clones were determined using 5'/3' RACE techniques and designated *GtMYBP3* and *GtMYBP4*.

Phylogenetic analysis showed that *GtMYBP3* and *GtMYBP4* belonged to P1/subgroup 7 (Fig. 2). The *GtMYBP3* cDNA (accession no. AB733016) was 1474 bp in length and encoded a protein of 329 amino acid residues, whereas the *GtMYBP4* cDNA (accession no. AB289446) was 1303 bp encoded a 376 amino acid protein. The deduced amino acid sequence of *GtMYBP3* was 79.8% identical to that of *GtMYBP4* in the R2R3-MYB DNA-binding domain, and 43.5% identical overall (Supplementary Fig. S1). *GtMYBP3* was 84.6, 89.4, and 83.7% identical to *Arabidopsis* AtMYB12, grape *VvMYBF1*, and maize *ZmP1*, respectively, whereas *GtMYBP4* was 78.8, 80.8, and 79.8% identical to those three genes, respectively. Although the sequence similarity between R2R3-MYB proteins is generally restricted to the

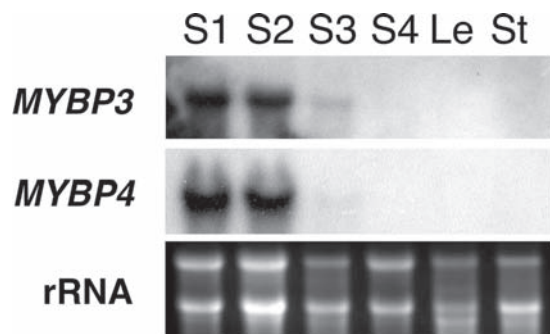
N-terminus, the SG7 motif (GRTxRSxMK; Stracke *et al.*, 2001) and the SG7-2 motif ([W/x][L/x]LS; Czemmell *et al.*, 2009) are characteristic of R2R3-MYB proteins regulating flavonol biosynthesis. Both SG7 motifs were partially conserved in *GtMYBP3* and *GtMYBP4*. *GtMYBP3* and *GtMYBP4* shared 56% identity (five out of nine amino acid residues) with the SG7-1 motif and 75% (3/4) and 50% (2/4) identity with the SG7-2 motif, respectively (Supplementary Fig. S1).

#### Expression profiles of *GtMYBP3* and *GtMYBP4* transcripts in gentian

To reveal the temporal and spatial expression of *GtMYBP3* and *GtMYBP4* in gentian plants, northern blot analysis was performed using total RNA isolated from petals (at four developmental stages), leaves, and stems (Fig. 3). *GtMYBP3* and *GtMYBP4* transcripts were detected at the early flower development stages prior to pigmentation, but disappeared during later



**Fig. 2.** Phylogenetic analysis of deduced amino acid sequences of R2R3-MYB transcription factors in higher plants. The phylogenetic tree of the R2R3-MYB domain was generated using MEGA software (Tamura *et al.*, 2007). Numerals next to branch nodes indicate bootstrap values from 1000 replications. The bar indicates an evolutionary distance of 0.05%. Accession numbers in the GenBank/EMBL/DBJ database are as follows: gentian: MYBP3 (AB733016), MYBP4 (AB289446), MYBP5 (AB733616), MYBP6 (AB733617), MYBP8 (AB733618), MYBP9 (AB733619), and MYB3 (AB289445); *Arabidopsis*: MYB11 (NM\_116126), MYB12 (DQ224277), MYB111 (NM\_124310), TT2, (AJ299452), PAP1 (AF325123), and PAP2 (AF325124); *gerbera*: MYB1 (AJ554697); grape: MYBF1 (FJ948477), and MYBA1-1 (AB073010); maize: P1 (M73028), P2 (AF210616), C1 (MZEMYBAA), and PL (AF015268); *petunia*: mybPh1 (Z13996) and AN2 (AF146702); snapdragon, MIXTA (X79108), VENOSA (DQ275531), ROSEA1 (DQ275529), and ROSEA2 (DQ275530); sorghum: Y1 (AY860968); and tomato: SIMYB12 (EU419748).



**Fig. 3.** Accumulation profiles of *GtMYBP3* and *GtMYBP4* transcripts in gentian plants. Northern blot analysis was performed using total RNA isolated from petal samples at four different flower developmental stages (S1–S4) and from leaf (Le) and stem (St) samples. RNA was blotted onto nylon membrane and hybridized with DIG-labelled *GtMYBP3* or *GtMYBP4* probes. Ethidium bromide-stained rRNA bands are shown as loading controls.

flower pigmented stages. Therefore, *GtMYBP3* and *GtMYBP4* would not be expected to regulate anthocyanin accumulation in gentian flowers. They could not be detected in vegetative tissues, such as leaves and stems. These expression profiles coincide with the accumulation profiles of flavones, which are abundantly present in young flower petals. The expression of *GtFNSII* and *GtF3'H* genes, which are involved in flavone biosynthesis, were also detected at early flower developmental stages (Nakatsuka *et al.*, 2005). Therefore, the temporal and spatial profiles of *GtMYBP3* and *GtMYBP4* transcripts correlated with those of flavone biosynthesis.

#### Protein–protein interactions between *GtMYBs* and *GtbHLH1*

To investigate protein–protein interactions, this study employed a yeast two-hybrid system. A preliminary experiment showed that GAL4 DNA-binding domain-fused *GtMYBs* all led to false-positive results; therefore, the interaction among *GtMYB* proteins could not be revealed in this study. *GtbHLH1*, a transcription factor regulating the anthocyanin biosynthetic pathway, interacted with *GtMYB3* (Supplementary Fig. S2). However, neither *GtMYBP3* nor *GtMYBP4* interacted with *GtbHLH1* in this study (Supplementary Fig. S2). Therefore, it was assumed that the activities of *GtMYBP3* and *GtMYBP4* were independent of the bHLH co-activator, as well as ZmP1.

#### Activation ability of *GtMYBP3* and *GtMYBP4* on promoters of flavonoid-biosynthetic genes by transient expression assay

To gain an insight into the activation ability of *GtMYBP3* and *GtMYBP4* on flavonoid biosynthesis, a transient expression assay in protoplasts of cultured *Arabidopsis* T87 suspension cells was used. About 1 kb of the 5'-upstream sequences of *GtCHS* (Kobayashi *et al.*, 1998), *GtF3'5'H* (Nakatsuka *et al.*, 2008b), *GtFNSII*, and *GtF3'H* were isolated from the gentian genome and connected to a reporter firefly luciferase (LUC) gene.

*GtCHSpro*, *GtFNSIIpro*, and *GtF3'Hpro* contained the binding sequences of vertebrate MYB protein ( $C^T/AACT^T/G$ , black box), a P-recognition element ( $CC^T/AACC$ , diagonal box; Grotewold *et al.*, 1994), and an ACGT-containing element (CACGT; Hartmann *et al.*, 1998), as shown in Fig. 4A. By contrast, *GtF3'5'Hpro* contained the vertebrate MYB protein and P-recognition element, but not the ACGT-containing element (Nakatsuka *et al.*, 2008b).

*GtMYBP3* had about 2-, 5-, and 120-fold activation ability on *GtCHS*, *GtFNSII* and *GtF3'H* promoters, respectively, compared with the vector control (Fig. 4B). Similarly, *GtMYBP4* also enhanced *GtFNSII* and *GtF3'H* promoter activity, but its ability was weaker than *GtMYBP3*. Conversely, neither *GtMYBP3* nor *GtMYBP4* could induce *GtF3'5'H* promoter activity. No synergistic effect of *GtMYBP3* and *GtMYBP4* was observed in the transient expression analysis (Supplementary Fig. S3).

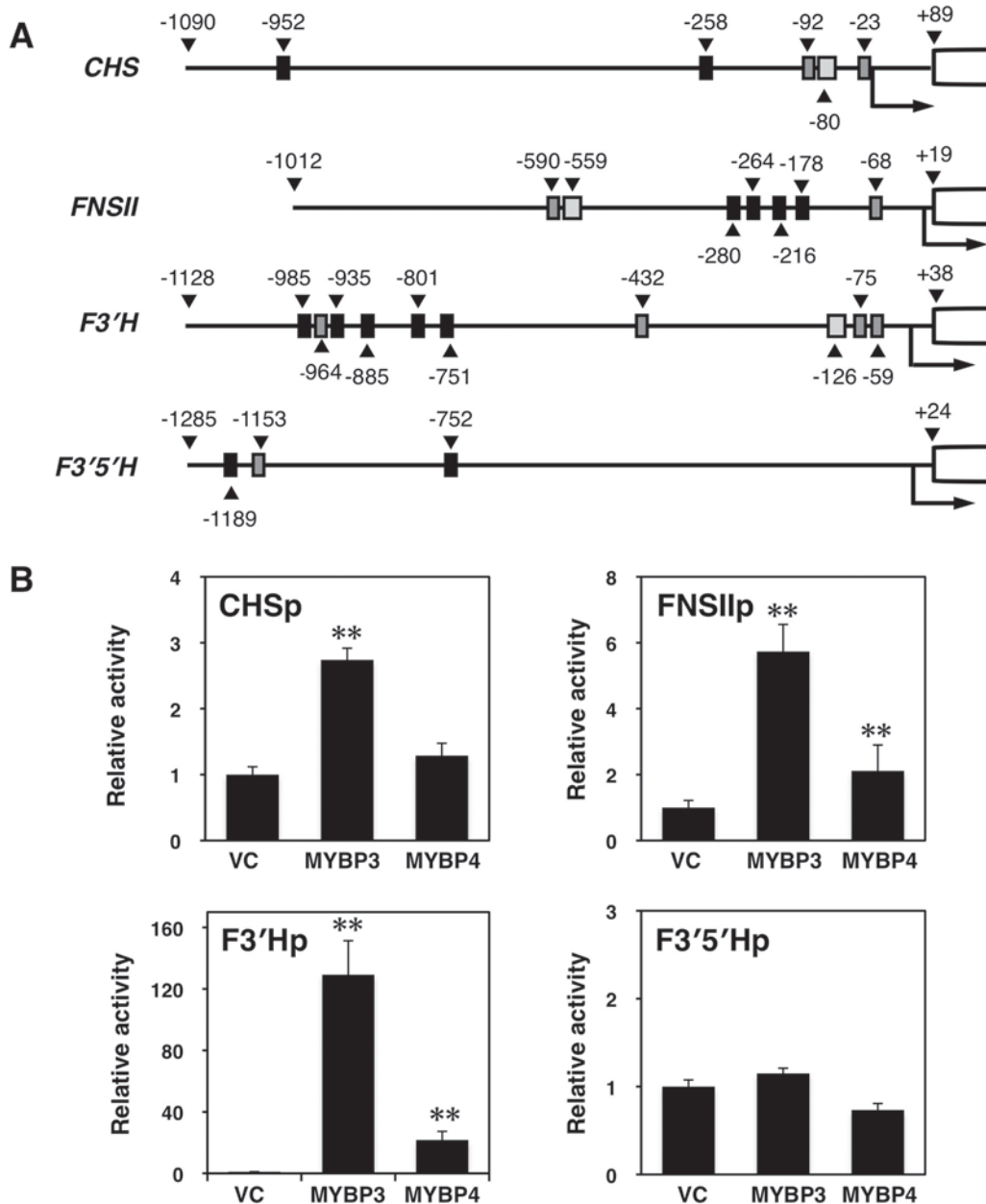
#### *GtMYBP3* and *GtMYBP4* overexpression in transgenic *Arabidopsis* plants

To confirm whether the *GtMYBP3* and *GtMYBP4* genes can regulate early flavonoid biosynthesis *in planta*, this study produced transgenic *Arabidopsis* plants expressing *GtMYBP3* and *GtMYBP4* under the control of a constitutive CaMV35S promoter.

Both constructs were transformed into *Arabidopsis* plants using the floral dip method, and two independent T<sub>2</sub> lines were produced, respectively. No morphological changes were observed in *GtMYBP3*- and *GtMYBP4*-expressing *Arabidopsis* plants compared with vector control plants (data not shown). Flavonoid accumulation of transgenic *Arabidopsis* seedlings was visualized with DPBA and imaged by epifluorescence microscopy (Fig. 5). In wild-type and vector control plants, cotyledons showed intense orange fluorescence implying abundant accumulation of flavonol derivatives. Their hypocotyls and roots showed yellow and blue fluorescence implying some flavonol accumulation. However, *GtMYBP3*- and *GtMYBP4*-expressing *Arabidopsis* showed intense orange fluorescence over the entire plant.

In both *GtMYBP3*- and *GtMYBP4*-expressing transgenic seedlings, the expression levels of each transgene in clone no. 1 was approximately 2-fold higher than in clone no. 2 (Supplementary Fig. S4A). Among genes involved in phenylpropanoid pathway, the expression levels of *AtPAL1* and *AtPAL2* were significantly enhanced in transgenic plants. *AtPAL1* and *AtPAL2* transcripts were upregulated by 1.4–1.8-fold in both *GtMYBP3*- and *GtMYBP4*-expressing plants compared with the vector control (Fig. 6). Induction of *Arabidopsis* 4-coumarate:CoA ligase (*At4CL3*) expression, which contributes to flavonoid biosynthesis, was observed during overexpression of *GtMYBP3* (2.8-fold) and *GtMYBP4* (7.3-fold). In *GtMYBP3*-expressing transgenic seedlings, the expression levels of several genes, including *AtPAL4*, *At4CL2*, and *At4CL5*, were reduced in both lines.

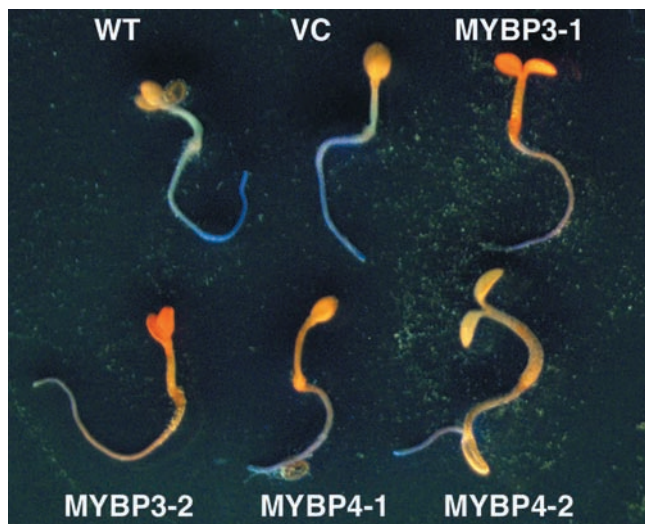
With regard to flavonoid biosynthetic genes, the expression of *AtCHS*, *AtCHI*, *AtF3H* and *AtF3'H* were upregulated by 6.3-, 2.7-, 3.2-, and 3.0-fold, respectively, in *GtMYBP3*-expressing *Arabidopsis* plants compared with the vector control. Conversely, the overexpression of *GtMYBP4* induced the expression of



**Fig. 4.** 5'-flanking sequences of flavonoid biosynthetic genes from gentian and transient expression assay. (A) Structures of the promoters of *GtCHS*, *GtFNSII*, *GtF3'H*, and *GtF3'5'H*. Filled, striped, and dotted boxes indicate MYB recognition elements, vertebrate MYB (Urao et al., 1993), P-recognition element (Grotewold et al. 1994), and an ACGT-containing element (ACE, Hartmann et al., 1998, 2005), respectively. The positions upstream of the transcription initiation site (arrow) are indicated as numerals above the filled triangles. The coding regions are indicated as open boxes. (B) Effect of *GtMYBP3* and *GtMYBP4* on promoter activities of four flavonoid biosynthetic genes. Transient expression assays were performed by transfecting the reporter and effector plasmid DNA into the protoplasts from *Arabidopsis* T87 cells. *GtCHS*pro-LUC, *GtFNSII*pro-LUC, *GtF3'H*pro-LUC, or *GtF3'5'H*pro-LUC were used as reporters, and p35Spro-*GtMYBP3*, p35Spro-*GtMYBP4*, or pBI221 (negative control) were used as the effector. p35Spro-RLUC was also used as a transformation control. The promoter activation activities are indicated as relative values compared with that of the negative control. Asterisks indicate statistically significant differences between the means for negative control (pBI221) and tested genes, as judged by Student's *t*-test ( $P < 0.01$ ).

*AtCHS*, *AtCHI*, and *AtFLS* transcripts by 6.3-, 4.8-, and 2.9-fold. The *AtF3H* and *AtF3'H* genes were also induced in *GtMYBP4*-expressing *Arabidopsis* line no. 2. The expression of *AtDFR*, encoding an enzyme of a later step of the anthocyanin biosynthetic pathway, was suppressed in *GtMYBP3*-expressing plants,

whereas no difference was detected in *GtMYBP4*-expressing plants. Expression of endogenous flavonol-specific R2R3-MYB genes, including *AtMYB12*, *AtMYB11*, and *AtMYB111*, did not markedly change in *GtMYBP3* and *GtMYBP4*-expressing seedlings (Supplementary Fig. S5). Thus, the overexpression of



**Fig. 5.** Visualized flavonoid accumulation in transgenic *Arabidopsis* seedlings. Flavonoid staining in wild type (WT), vector control (VC), and transgenic seedlings. Two independent transgenic lines are shown. Norflurazon-bleached seedlings were stained with diphenylboric acid 2-aminoethyl ester and photographed under UV light.

*GtMYBP3* and *GtMYBP4* in *Arabidopsis* enhanced or decreased the expression of several genes encoding enzymes of the phenylpropanoid and flavonoid biosynthetic pathways. However, there was a slight difference in the affected gene sets and intensities between *GtMYBP3* and *GtMYBP4*.

#### *GtMYBP3* and *GtMYBP4* overexpression in transgenic tobacco plants

As shown above, transgenic *Arabidopsis* showed clear changes in flavonoid biosynthesis in vegetative tissues. However, flavonoid compositions, including anthocyanin pigments, were difficult to analyse in the floral tissues. Thus, to investigate the effect of *GtMYBP3* and *GtMYBP4* overexpression on floral flavonoid biosynthesis, this study produced transgenic tobacco plants. The petals of tobacco accumulate flavonol and anthocyanin derivatives (Nakatsuka *et al.*, 2007). Tobacco leaf sections were transformed by *A. tumefaciens* harbouring the same binary vector construct used for *Arabidopsis* transformation. Over 20 independent transgenic lines were grown in a greenhouse, and the T<sub>1</sub> seeds were collected after self-pollination. Two representative lines for each construct were chosen and subjected to further analysis.

The pigmentation of the petals of *GtMYBP3*- and *GtMYBP4*-expressing tobacco plants was decreased compared with the wild type (Fig. 7A). Anthocyanin levels in *GtMYBP3*-expressing transgenic tobacco petals were 22–46% lower compared with the wild type (Fig. 7B). However, the amounts of flavonol were the same between wild-type and *GtMYBP3*-expressing plants (Fig. 7C). Anthocyanin levels in *GtMYBP4*-expressing transgenic tobacco petals were 25–28% lower compared with wild type (Fig. 7B). In addition, the amounts of flavonols were increased by 1.5–2.6-fold in the petals of *GtMYBP4*-expressing

tobacco plants (Fig. 7C) and were inversely correlated with the accumulation levels of anthocyanins. No difference in flavonol and anthocyanin components was observed between any transgenic and wild-type petals by HPLC analysis (data not shown).

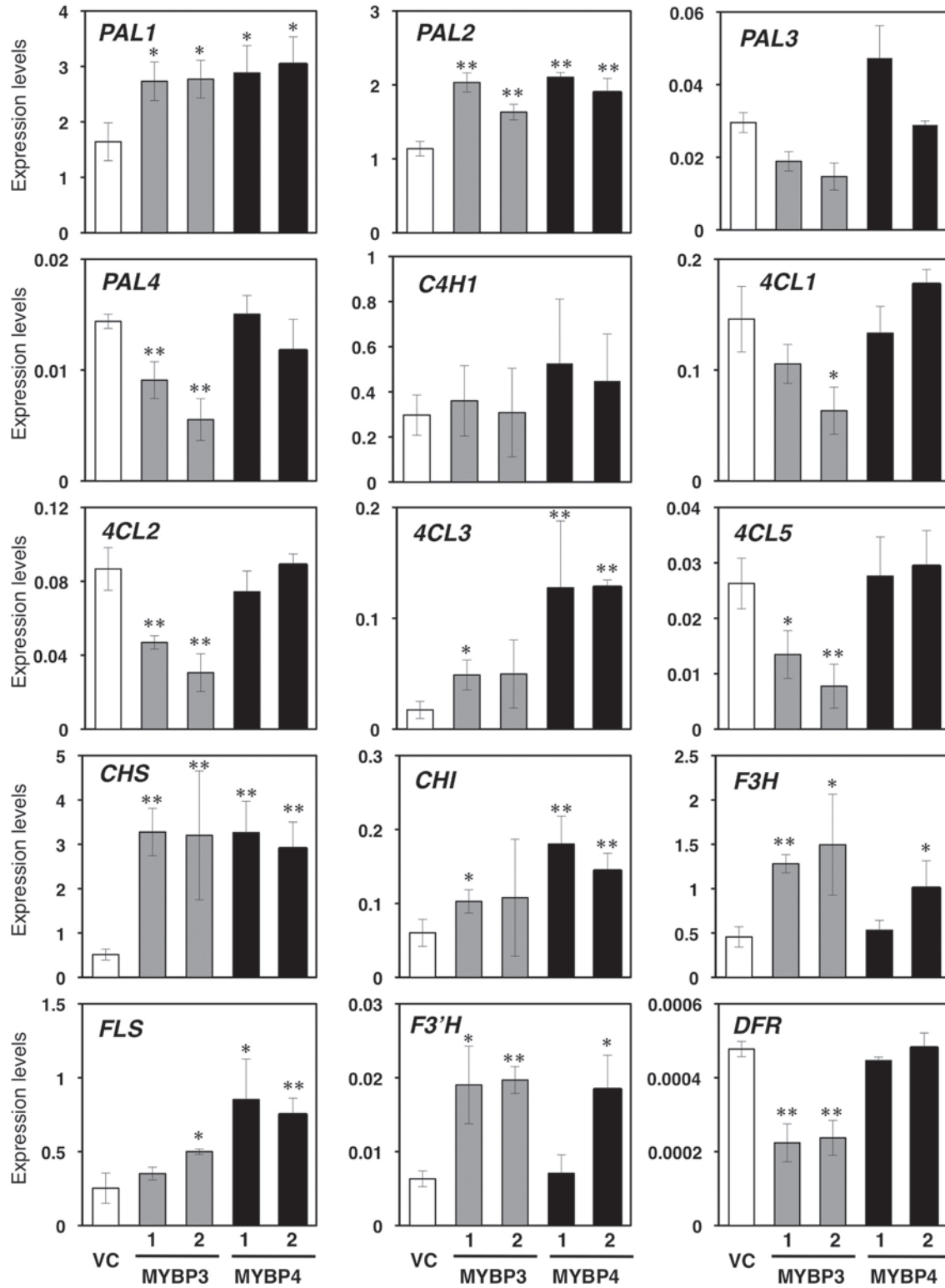
The expression of the transgenes were confirmed (Supplementary Fig. S4B). Expression of *NtCHS*, *NtCHI*, *NtF3'H*, and *NtFLS* was enhanced 2.1-, 3.5, 6.0-, and 5.1-fold in the petals of *GtMYBP4*-expressing transgenic plants (Fig. 7D). The expression of the *NtF3H* gene decreased in *GtMYBP4*-expressing plants. However, no change was detected in the *NtDFR* gene, which is involved in late flavonoid biosynthesis. On the other hand, no significant change in expression of flavonoid biosynthetic genes was observed in *GtMYBP3*-expressing tobacco plants (Fig. 7D).

## Discussion

A previous study revealed that two transcription factors, GtMYB3 and GtHLH1, regulate gentiodelphin biosynthesis in the petals of Japanese gentian (Nakatsuka *et al.*, 2008b). The complex of GtMYB3 and GtHLH1 proteins activated the expression of genes encoding enzymes involved in the anthocyanin biosynthetic pathway after the *GtF3H* gene, but could not induce the transcripts of early flavonoid biosynthetic genes. Recently, some transcription factors controlling early flavonoid biosynthesis, especially flavonol accumulation, have been reported in several plant species, including *Arabidopsis* seedlings (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007), grapes (Czemmel *et al.*, 2009), and tomatoes (Ballester *et al.*, 2010). However, no transcription factor controlling early flavonoid biosynthesis in the petals has been identified.

In this study, two R2R3-MYB transcription factors from the petals of Japanese gentian were identified. GtMYBP3 and GtMYBP4 were classified into P1/subgroup 7 (Fig. 2), which were reported to regulate early flavonoid biosynthesis (Dubos *et al.*, 2010). *Arabidopsis* R2R3-MYB subgroup 7 contains AtMYB12/PFG2, AtMYB11/PFG1, and AtMYB111/PFG1, and these are known to control flavonol biosynthesis individually in the different organs (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007; Dubos *et al.*, 2010). The deduced amino acid sequence of GtMYBP3 is highly similar to GtMYBP4 in the R2R3-MYB DNA-binding domain (79.8%), suggesting that they might be functionally redundant genes (Fig. 2 and Supplementary Fig. S1). SG7 and SG7-2 motifs are conserved in the C-termini of R3R3-MYBs belonging to subgroup 7 in *Arabidopsis* and grapevine (Stracke *et al.*, 2001; Czemmel *et al.*, 2009; Dubos *et al.*, 2010). However, the SG7 or SG7-2 motifs were not conserved in tomato SIMYB12, which has been identified as a flavonol regulator in tomato fruits (Ballester *et al.*, 2010). The SG7 and SG7-2 motifs of GtMYBP3 and GtMYBP4 also had amino acid substitutions at some positions (Supplementary Fig. S1). Therefore, conservation of SG7 motifs in GtMYBP3 and GtMYBP4 might not be so important for regulation of early flavonoid biosynthesis in gentian petals.

*GtMYBP3* and *GtMYBP4* had similar temporal and spatial expression profiles, expressing strongly during early developmental stages (stages 1 and 2) of flower petals in gentians

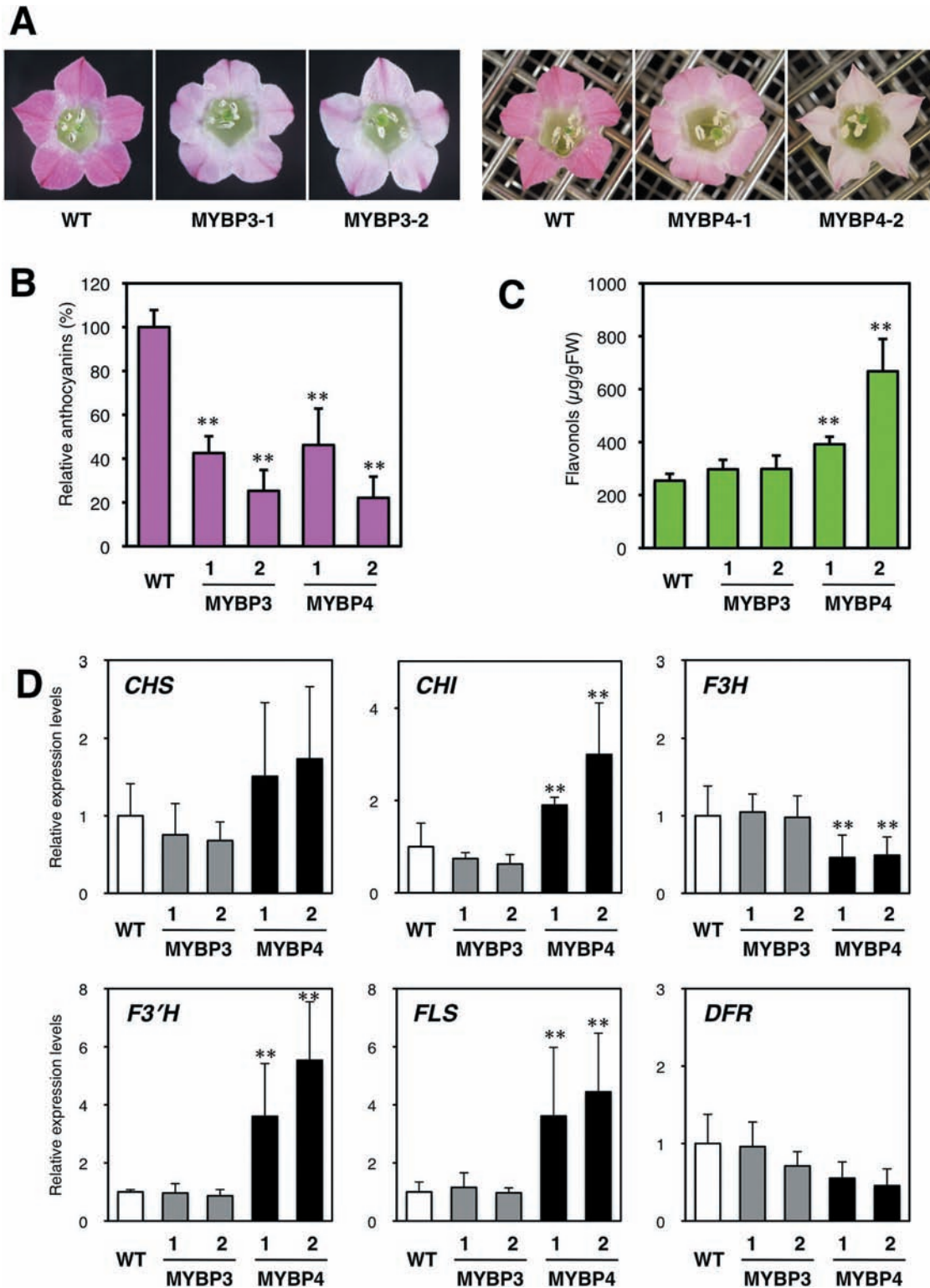


**Fig. 6.** Expression analysis of endogenous flavonoid biosynthetic genes in transgenic *Arabidopsis*. The effects of *GtMYBP3* and *GtMYBP4* overexpression on endogenous phenylpropanoid and flavonoid biosynthetic genes were investigated using qRT-PCR analyses in vector control (VC) and 5-day-old transgenic seedlings. Two independent transgenic lines shown in Fig. 5 were analysed. Asterisks indicate statistically significant differences between the means for vector control and transgenic lines, as judged by Student's *t*-test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

(Fig. 3). These expression profiles corresponded well with the accumulation profiles of *FNSII* and *F3'H* transcripts and flavone derivatives (Nakatsuka et al., 2005). In grapevine, *VvMYBF1*

transcripts were detected during flowering and in skins of ripening berries and were correlated with the accumulation of flavonol and expression of *VvFLS1* (Czemmel et al., 2009).





**Fig. 7.** Phenotype and flavonoid analysis in transgenic tobacco flowers. (A) Typical flower phenotypes of wild-type (WT) and *GtMYBP3*- and *GtMYBP4*-expressing transgenic tobacco plants. Two independent transgenic lines per construct are shown. (B) Anthocyanin concentrations of WT and *GtMYBP3*- and *GtMYBP4*-expressing transgenic petals. (C) Flavonol concentrations of WT and *GtMYBP3*- and *GtMYBP4*-expressing transgenic petals. (D) Expression analyses of endogenous flavonoid biosynthetic genes in transgenic flowers. The effects of *GtMYBP3* and *GtMYBP4* overexpression on endogenous flavonoid biosynthetic genes were investigated using qRT-PCR analyses in WT and transgenic petals. Asterisks represent statistically significant differences between the means for wild-type and transgenic lines, as judged by Student's *t*-test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).

Transient expression in *Arabidopsis* suspension cells showed that both GtMYBP3 and GtMYBP4 could enhance the promoter activities of gentian *GtFNSII* and *GtF3'H*, which encode enzymes of the early flavonoid biosynthesis pathway (Fig. 4B). GtMYBP3 also enhanced the promoter activity of gentian *GtCHS*. However, the promoter activity of *GtF3'5'H*, encoding an enzyme of the late flavonoid biosynthesis pathway, could not be activated by either GtMYBP3 or GtMYBP4. Nakatsuka *et al.* (2008b) demonstrated that GtMYB3 and GtbHLH1, which are anthocyanin biosynthetic regulators in gentian flowers, induced promoter activity of *GtF3'5'H*, but not of *GtCHS*. These results suggested that GtMYBP3 and GtMYBP4 control the expression of early flavonoid biosynthetic genes, unlike GtMYB3 and GtbHLH1. For the promoters of all early flavonoid biosynthetic genes, the activation intensities of GtMYBP3 were higher than those of GtMYBP4 (Fig. 4B). The *GtFNSII* promoter was also activated by GtMYBP3 or GtMYBP4 in a transient assay using gentian mesophyll protoplasts, whereas *GtCHS* was activated by GtMYBP3 only (Supplementary Fig. S6), confirming the results of transient expression assays in *Arabidopsis* suspension cells (Fig. 5B). The reason for the different activation ability between GtMYBP3 and GtMYBP4 is not yet clearly understood, but it might depend on the different DNA-binding activities on each promoter and/or on interactions with other transcription factor(s) to regulate flavonoid biosynthesis. Two *cis*-elements, a P-recognition element and an ACGT-containing element, were present in close proximity to the transcription initiation site of the promoters of *GtCHS*, *GtFNSII*, and *GtF3'H*, but not *GtF3'5'H* (Fig. 4A). The P-recognition element has been identified as a *cis*-binding site for the maize P1 protein, controlling 3-deoxyanthocyanin phlobaphene and C-glycosyl flavone maysin biosynthesis (Grotewold *et al.*, 1994). Therefore, GtMYBP3 and GtMYBP4 might bind to the P-recognition element and activate the transcription of downstream genes. This is reasonable, because yeast two-hybrid analysis also showed that the GtMYBP3 or GtMYBP4 proteins did not interact with GtbHLH1 (Supplementary Fig. S2). Moreover, ACGT-containing element potentially binds to bZIP transcription factors, which work together with R2R3-MYB in light-inducible pigmentation (Hartmann *et al.*, 2005). Further analysis is necessary to elucidate the functions of GtMYBP3 and GtMYBP4 in relation to other transcription factors, including bZIP.

Overexpression of *GtMYBP3* and *GtMYBP4* led to flavonol accumulation in transgenic *Arabidopsis* seedlings (Fig. 5). Overexpression of *AtMYB12* also resulted in increased flavonol amounts in *Arabidopsis* plants (Mehrtens *et al.*, 2005). qRT-PCR analyses of endogenous phenylpropanoid and flavonol biosynthetic genes showed the activated endogenous gene sets differed somewhat between *GtMYBP3*- and *GtMYBP4*-expressing *Arabidopsis* (Fig. 6). The expression levels of three endogenous flavonol-specific transcription factor genes, *AtMYB12*, *AtMYB11*, and *AtMYB111*, were not markedly affected in the transformants and there was no relationship between the expression levels of foreign genes and endogenous genes (Supplementary Figs. S4A and S5); therefore, an indirect effect via activation of endogenous transcription factors was excluded. GtMYBP3 activated the transcripts of *AtPAL1*, *AtPAL2*, *At4CL3*, *AtCHS*, *AtCHI*, *AtF3H*, and *AtF3'5'H*, whereas GtMYBP4 enhanced *AtPAL1*, *AtPAL2*, *At4CL3*, *AtCHS*, *AtCHI*, and *AtFLS* transcripts in

transgenic *Arabidopsis* (Fig. 6). *Arabidopsis* genome comprises four *PAL* genes (*AtPAL1* to *AtPAL4*), among them *AtPAL1* and *AtPAL2* have a redundant role in flavonoid biosynthesis (Huang *et al.*, 2010). *4CL* also comprises a multigene family, *At4CL1*, *At4CL2*, *At4CL3*, and *At4CL5*, in *Arabidopsis* (Ehltting *et al.*, 1999; Costa *et al.*, 2005). It is notable that *At4CL3* is likely to participate in the biosynthetic pathway leading to flavonoids, whereas *At4CL1* and *At4CL2* are probably involved in lignin formation and in the production of additional phenolic compounds other than flavonoids (Ehltting *et al.*, 1999). Therefore, the observed activation of *AtPAL1*, *AtPAL2*, and *At4CL3* transcripts in transgenic *Arabidopsis* plants would be reasonable in light of the functions of GtMYBP3 and GtMYBP4. GtMYBP4 induced stronger expression of almost all phenylpropanoid and flavonol biosynthetic genes, except for *AtF3H* and *AtF3'5'H*, than GtMYBP3 (Fig. 6). *AtF3'5'H* was not activated by AtMYB12 or ZmP1 (Mehrtens *et al.*, 2005). The differences in the controlled gene sets between GtMYBP3 and GtMYBP4 might reflect the differences in their C-terminal regions. ZmP1 activates the promoters of *AtCHS*, *AtF3H*, and *AtFLS*, although *AtFLS* induction was only 18% of that observed for AtMYB12 (Mehrtens *et al.*, 2005). Thus, the intensity of transcriptional activation for individual flavonol biosynthetic genes seems to be different among P1 orthologues. The suppression of several biosynthetic genes in the *GtMYBP3*-expressing transgenic *Arabidopsis* seedlings also suggested that other phenylpropanoid metabolism, such as lignins, organic acids, and proanthocyanidins, were regulated; therefore, detailed analyses are necessary in the future.

To further investigate the functions of GtMYBP3 and GtMYBP4 in flowers, they were expressed in tobacco plants, which are well known for flavonoid biosynthesis in their petals (Nishihara *et al.*, 2005; Nakatsuka *et al.*, 2007, 2008a). The flowers of *GtMYBP3*- and *GtMYBP4*-expressing transgenic tobacco plants showed decreased colour intensity (Fig. 7A). The phenotype of *GtMYBP4*-expressing tobacco flowers arose from increased flavonol and reduced anthocyanin accumulation (Fig. 7B, C). qRT-PCR analysis also confirmed that *GtMYBP4* increased the expression levels of four endogenous tobacco genes, *CHI*, *F3'H*, and *FLS*, by 3–6-fold, but did not activate the expression of flavonoid biosynthesis genes, *F3H* and *DFR* (Fig. 7D). These results suggested the increased flavonol accumulation resulted from the activation of genes encoding enzymes catalysing the early steps in flavonoid biosynthesis, but not from the suppression of anthocyanin biosynthetic genes in transgenic tobacco plants. The inverse correlation between anthocyanin and flavonol levels in the flowers probably reflects competition between these two branches for flavonoid metabolites (Davies *et al.*, 2003). The overexpression of *AtMYB12*, a regulator of flavonol biosynthesis, induced the enhanced expression of *NtPAL*, *NtCHS*, *NtCHI*, and *NtFLS* and increased flavonol accumulation in tobacco petals (Luo *et al.*, 2008). Therefore, heterologous expression of *GtMYBP4* could control the early flavonoid biosynthesis in tobacco plants. Conversely, *GtMYBP3*-expressing tobacco flowers showed reduced anthocyanin accumulation, but showed no increase in flavonol accumulation (Fig. 7B, C). No significantly different expression levels of flavonol and anthocyanin biosynthetic genes were detected between wild-type and *GtMYBP3*-expressing plants (Fig. 7D). *Arabidopsis AtMYB4*

and strawberry *FaMYB1*, classified into subgroup 4, suppress the expression of cinnamate 4-hydroxylase and anthocyanidin synthase, respectively (Jin *et al.*, 2000; Aharoni *et al.*, 2001). Therefore, GtMYBP3 might affect the expression of genes that were not investigated in this study, such as the phenylpropanoid biosynthetic and flavonoid modification genes, in tobacco petals. Proanthocyanidins (condensed tannins) compounds also derived from the flavonoid biosynthetic pathway, and two key enzymes, anthocyanidin reductase (ANR) and leucoanthocyanidin reductase (LAR), are involved in their biosynthesis (Tanner *et al.*, 2003; Xie *et al.*, 2003). Recently, Han *et al.* (2012) reported that ectopic expression of apple *ANR* in tobacco led to downregulation of both *CHI* and *DFR* in the flowers, leading to loss of anthocyanin. Similarly, in some cases, metabolic engineering of the flavonoid biosynthetic pathway has been known to induce feedback suppression of endogenous related genes. Therefore, the reduced anthocyanin accumulation in *GtMYBP3*-expressing tobacco flowers might result from such unexpected regulation of genes encoding phenylpropanoid and flavonoid biosynthetic enzymes, although further metabolic and gene expression analyses are necessary to explain this observation. As shown above, the endogenous gene sets activated by GtMYBP3 and GtMYBP4 were different between transgenic *Arabidopsis* seedlings and tobacco flowers (Figs. 6 and 7). However, the targeted gene sets of GtMYBP3 and GtMYBP4 mostly overlapped in the early flavonoid biosynthetic genes; therefore, the functions of these two genes were thought to be complementary in gentian flowers. Moreover, the differences in the targeted gene sets among plant hosts (*Arabidopsis* and tobacco) suggested that full activation of GtMYBP3 and GtMYBP4 transcription factors probably required cofactors, such as bZIP (Hartmann *et al.*, 2005).

Petunia flowers have been extensively studied as models for flavonoid biosynthesis and are proposed as a model showing that early- and late-biosynthetic genes are controlled by two different regulators (Quattrocchio *et al.*, 1993). However, although it has been revealed that AN1/AN2 regulate the expression of anthocyanin biosynthetic genes, no transcription factor involved in the flavonol biosynthetic pathway has been identified. Uniquely, GhMYB1 was isolated from the petals of *Gerbera hybrida*, but its function has not been characterized (Elomaa *et al.*, 2003). Therefore, *GtMYBP3* and *GtMYBP4* are the first characterized transcription factors that are involved in the early flavonoid biosynthesis in petal organs. Their identification will advance the understanding of flavonoid biosynthesis in floricultural crops.

## Supplementary material

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** Alignment of P1 orthologue proteins in higher plants.

**Supplementary Fig. S2.** Yeast two-hybrid analysis to examine the protein–protein interaction between GtMYBs and GtbHLH1.

**Supplementary Fig. S3.** Effect of co-transfection of GtMYBP3 and GtMYBP4 on promoter activities of *GtCHS*, *GtFNSII*, and *GtF3H*.

**Supplementary Fig. S4.** Confirmation of the expression of transgenes in transgenic *Arabidopsis* and tobacco.

**Supplementary Fig. S5.** Expression analyses of endogenous flavonol-specific transcription factor genes in transgenic *Arabidopsis*.

**Supplementary Fig. S6.** Transient expression assay in gentian mesophyll protoplast.

**Supplementary Table. S1.** Degenerate PCR, probe, and inverse PCR primers used in this study.

**Supplementary Table. S2.** Primers used for quantitative RT-PCR analysis.

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