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Identification and Characterization of R2R3-MYB and bHLH Transcription Factors Regulating Anthocyanin Biosynthesis in Gentian Flowers

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Gentian plants have vivid blue-colored flowers, caused by accumulation of a polyacylated anthocyanin 'gentiodelphin'. We previously performed expression analysis of gentiodelphin biosynthetic genes, and hypothesized that the white-flowered gentian cultivar 'Polarno White' might have resulted from the mutation of certain regulatory factors responsible for anthocyanin biosynthesis in flower petals. In this study, we isolated 26 *R2R3-MYB* gene fragments including four full-length cDNAs (*GtMYB2a*, *GtMYB2b*, *GtMYB3* and *GtMYB4*) and one basic helix–loop–helix (*bHLH*) gene (*GtbHLH1*) from blue-flowered gentian by degenerate PCR and rapid amplification of cDNA ends (RACE). Phylogenetic tree analysis showed that GtMYB3 was categorized into a clade involved in anthocyanin biosynthesis including petunia AN2 and Arabidopsis PAP1. On the other hand, *Gt*bHLH*1* exhibited high identity with petunia AN1 based on both phylogenetic and genomic structural analyses. Temporal profiles of *GtMYB3* and *GtbHLH1* transcript levels corresponded well with those of gentiodelphin accumulation and their biosynthetic genes in petals. Yeast two-hybrid analysis showed that GtbHLH1 interacted with GtMYB3. Moreover, transient expression analysis indicated that the co-expression of *GtMYB3* and *GtbHLH1* could enhance the promoter activities of late anthocyanin biosynthetic genes in tobacco BY2 cells. We also revealed that in cv. 'Polarno White' the *GtMYB3* genes were mutated by insertions of transposable elements or uncharacterized sequences, indicating that the white coloration was caused by *GtMYB3* mutation. These results strongly suggested that GtMYB3 and GtbHLH1 are involved in the regulation of gentiodelphin biosynthesis in gentian flowers.

Keywords: Anthocyanin biosynthesis — bHLH — Gentian — R2R3-MYB — Transcription factor — White flower.

Abbreviations: 5AT, hydroxycinnamoyl-CoA:anthocyanin 5-*O*-acyltransferase; bHLH, basic helix–loop–helix; CHS, chalcone synthase; EBG, early biosynthetic gene; F3′5′H, flavonoid 3′,5′-hydroxylase; GUS, β-glucuronidase; LBG, late biosynthetic gene; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR; TIR, terminal inverted repeat; TSD, target site duplication; WDR, WD40 repeat.

The nucleotide sequences reported in this paper have been submitted to the GenBank database under accession numbers AB289443 (*GtMYB2a*), AB289444 (*GtMYB2b*), AB289445 (*GtMYB3*), AB289446 (*GtMYB4*), AB459661 (*GtbHLH1*), AB459662 (genomic *GtbHLH1*), AB378088 (*GtF3′5′H* promoter) and AB459663 (*Gt5AT* promoter).

Introduction

Gentian is one of the most popular floricultural plants in Japan, and has unique and brilliant blue-colored flowers. This flower color is known to be conferred by the accumulation of polyacylated anthocyanin [delphinidin 3-*O*-β-D-glucoside 5-*O*-(6-*O*-caffeoyl-β-D-glucoside) 3′-*O*-(6-*O*-caffeoyl-β-D-glucoside)], termed gentiodelphin, in flower petals (Goto et al. 1982). The chemical structure of gentiodelphin is thought to contribute to both blue color development and pigment stabilization by intramolecular sandwich-type stacking (Yoshida et al. 2000). Our previous research revealed that the accumulation of gentiodelphin increased during flower development and peaked at just before anthesis (Nakatsuka et al. 2005a). On the other hand, flavones, mainly apigenin and minor luteolin derivatives, also accumulated abundantly in gentian petals. Gentiodelphin biosynthesis has been well studied, and the genes encoding its biosynthetic enzymes have been extensively isolated and characterized (Tanaka et al. 1996, Fujiwara et al. 1998, Kobayashi et al. 1998, Fukuchi-Mizutani et al. 2003, Nakatsuka et al. 2005a, Nakatsuka et al. 2008, see also

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review by Nishihara et al. 2008). Flavonoid biosynthetic structural genes in gentian were classified into three groups based on their transcription profiles during flower development (Nakatsuka et al. 2005a, Nakatsuka et al. 2008). The first group (group 1) contains chalcone synthase (*CHS*) and chalcone isomerase (*CHI*), encoding enzymes common to flavone and gentiodelphin biosyntheses. The second group (group 2) contains flavone synthase II (*FNSII*) and flavonoid 3′-hydroxylase (*F3′H*), which are involved in the biosynthesis of flavones and cyanidin. The last group (group 3) contains genes encoding flavanone 3-hydroxylase (F3H), flavonoid 3′,5′-hydroxylase (F3′5′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), UDP-glucose: anthocyanidin 3-*O*-glucosyltransferase (3GT), UDP-glucose:anthocyanin 5-*O*-glucosyltransferase (5GT), hydroxycinnamoyl-CoA:anthocyanin 5-*O*-acyltransferase (5AT) and UDP-glucose:anthocyanin 3′-*O*-glucosyltransferase (3′GT), all of which catalyze reactions in anthocyanin biosynthesis. Indeed, in the different species studied so far, many flavonoid structural genes have been found to be co-regulated (Mol et al. 1998, Koes et al. 2005, Quattrocchio et al. 2006). In Arabidopsis, two types of co-regulated structural genes can be distinguished in the seedling. One type is early biosynthetic genes (EBGs) that are common to the different flavonoid subpathways, and the other is late biosynthetic genes (LBGs), which are involved in proanthocyanin and anthocyanin biosyntheses (Pelletier et al. 1999, Nesi et al. 2000). The patterns in other dicots such as snapdragon (*Antirrhinum majus*) and petunia (*Petunia hybrida*) are also similar (Martin et al. 1991, Quattrocchio et al. 1993, Quattrocchio et al. 1998). The transcription factor genes regulating flavonoid biosynthesis are currently known to belong to three distinct gene families, containing R2R3-MYB, basic helix–loop–helix (bHLH) and WD40 repeats (WDRs) (Koes et al. 2005, Quattrocchio et al. 2006). In Arabidopsis, three R2R3-MYBs, AtMYB11, AtMYB12 and AtMYB111, which act redundantly, are known to regulate the expression of EBGs and flavonol accumulation in the seedlings (Mehrtens et al. 2005, Stracke et al. 2007). On the other hand, the expression of LBGs is regulated by a regulatory complex composed of R2R3-MYB, bHLH and WDR (Baudry et al. 2004). In maize, R2R3-MYB proteins COLORED ALEURONE1 (C1) and PURPLE PLANT (P1), bHLH protein RED1 (R1) and BOOSTER1 (B1) are involved in anthocyanin pigmentation (Chandler et al. 1989, Goff et al. 1992). In Arabidopsis, it is known that *PRODUCTON OF ANTHOCYANIN PIGMENTS1* (*PAP1*)/*PAP2* and *TRANSPARENT TESTA2* (*TT2*), encoding R2R3-MYB proteins, control anthocyanin biosynthesis in seedlings and proanthocyanin in seeds, respectively. These Arabidopsis R2R3-MYBs also require bHLH protein, GLABRA3 (GL3), ENHANCER OF GLABRA3 (EGL3) and TT8, for full activation (Zhang et al. 2003, Zimmermann et al. 2004). In proanthocyanin biosynthesis in Arabidopsis seed coats, the WDR protein TRANSPARENT TESTA GLABRA1 (TTG1) interacts with TT8 but not TT2, and the ternary complex directly regulates the expression of *BANYULS* (*BAN*) (Baudry et al. 2004, Hartmann et al. 2005, Baudry et al. 2006). The MYB–bHLH–WDR protein complex plays a role not only in regulating anthocyanin biosynthesis but also in generating plant epidermal cellular diversity (Ramsay and Glover 2005). Advanced studies of the flower pigmentations of floricultural plants have been mainly carried out in petunia and snapdragon (Martin et al. 1991, Quattrocchio et al. 1993). Recessive white-flowered mutants of petunia plants have been shown to be caused by the insertion of the transposable element *dTph1* into *ANTHOCYANIN1* (*AN1*) and *AN2*, encoding bHLH and R2R3-MYB proteins, respectively (Quattrocchio et al. 1999, Spelt et al. 2000). AN2 interacted with AN1 and JAF13, which encodes another bHLH protein, and activated the expression of anthocyanin biosynthetic genes in petunia (Spelt et al. 2000). In addition to the AN1 and AN2 proteins, the cytoplasmic WDR protein AN11 is also required for anthocyanin accumulation, probably via post-translational regulation of MYB transcription factors (de Vetten et al. 1997). In Japanese morning glory (*Ipomoea nil*), the recessive mutant of *InMYB1*, encoding R2R3-MYB, exhibits white flowers with red stems and colored seeds, and that of *InWDR1*, encoding WDR, exhibits white flowers with green stems and ivory seeds (Morita et al. 2006).

We have previously revealed that the pigmentation of gentian flowers is regulated temporally and spatially by the level of transcription of each corresponding flavonoid biosynthetic gene, and found two different mutations in two independent white-flowered cultivars in gentian (Nakatsuka et al. 2005a, Nakatsuka et al. 2005b). One was a structural gene- (*ANS*) deficient mutation, and the other seemed to be a mutation of certain regulatory factor(s) controlling the expression of genes involved in the later steps of flavonoid biosynthesis (group 3). However, no transcription factors involved in flavonoid biosynthesis in gentian flowers have been identified to date. Thus, this study aimed at the cloning and functional characterization of transcription factors regulating anthocyanin biosynthesis from gentian plants. Here, we demonstrated that *R2R3-MYB* and *bHLH* homologs, termed *GtMYB3* and *GtbHLH1*, are involved in gentiodelphin biosynthesis in gentian petals. Moreover, we found that independent white-flowered gentian cultivars contained several mutations in the *GtMYB3* gene, and this strongly supported the hypothesis that GtMYB3 regulates gentiodelphin biosynthesis in gentian. This is the first study concerning the cloning and functional analysis of transcription factors of anthocyanin biosynthesis in gentian plants.

Results

Cloning of R2R3-MYB transcription factors from gentian flowers

A PCR-based method was used to isolate *R2R3-MYB* transcription factor genes from gentian flowers, based on a conserved region encoding the DNA-binding domain of R2R3-MYB proteins in higher plants. This strategy has been successfully applied to maize (Rabinowicz et al. 1999), Arabidopsis (Romero et al. 1998) and orchid (Wu et al. 2003).

An amplified fragment of about 260 bp was obtained from *Gentiana triflora* cv. Maciry flower cDNA using newly designed primers. However, when the primers as described by Rabinowicz et al. (1999) were used, a fragment of approximately 180 bp was amplified, which was the same length as previously reported. Sequence analysis revealed that the obtained fragments contained 26 groups of putative *R2R3-MYB* cDNA fragments (Supplementary Table S1, S2). Among them, the major clones were categorized into four groups and designated as the *GtMYB2a*, *GtMYB2b*, *GtMYB3* and *GtMYB4* genes. *GtMYB1* (accession No. D38165) was previously identified in the screening of a petal cDNA library (Kobayashi et al. 1998), but this gene was not amplified by the degenerate PCR strategy used in the current study. Although the deduced amino acid sequence of the *GtMYB2a* fragment corresponded completely with *GtMYB2b*, similarity at the nucleotide sequences level was only 87.5%. For these four *GtMYB* fragments, 5′- and 3′-RACE (rapid amplification of cDNA ends) technology was applied to isolate the corresponding full-length cDNAs. The deduced amino acid sequences of the four putative *GtMYB* genes were subjected to phylogenetic analysis with R2R3-MYB domains of proteins derived from other plant species. As shown in Fig. 1 and Supplementary Fig. S1, GtMYB3 and GtMYB4 were categorized into a group regulating flavonoid biosynthesis, while GtMYB1, GtMYB2a and GtMYB2b were categorized into other groups. GtMYB3 and GtMYB4 belonged to different R2R3-MYB subgroups for flavonoid biosynthesis. GtMYB3 exhibited 33.3, 33.9, 33.7, 37.9 and 31.0% identities with petunia AN2 (Quattrocchio et al. 1999), Arabidopsis PAP1, PAP2 (Borevitz et al. 2000) and TT2 (Nesi et al. 2001), and maize C1 (Paz-Ares et al. 1987), respectively, whereas GtMYB4 exhibited 31.2, 40.0, 36.3, 33.7 and 27.9% identities with Arabidopsis MYB11, MYB12 and MYB111 (Mehrtens et al. 2005, Stracke et al. 2007), maize P1 (Grotewold et al. 1994) and sorghum Y1 (Boddu et al. 2006), respectively. The former subclass plays a role in anthocyanin and proanthocyanin biosyntheses of their flowers, fruits, seeds and kernel pericarps, and the latter subclass plays a role in flavonol and phlobaphene biosyntheses of their seedlings and kernel pericarps. Thus, GtMYB3 is more likely to regulate anthocyanin biosynthesis in gentian petals.

On the other hand, GtMYB2a and GtMYB2b were similar to snapdragon MYB305, and Arabidopsis MYB24 and MYB2. Snapdragon MYB305 is reported to control phenylpropanoid biosynthesis (Moyano et al. 1996), while Arabidopsis MYB24 has a role in the anther development process (Yang et al. 2007). Arabidopsis MYB2 has been identified as an ABA-inducible transcription factor (Urao et al. 1993, Urao et al. 1996). Therefore, GtMYB2a and GtMYB2b were considered to be involved in the regulation of other biological phenomena such as morphogenesis or stress response rather than flavonoid biosynthesis; though further research will be necessary to reveal their functions. These results showed that GtMYB3 and GtMYB4 might be involved in the regulation of flavonoid biosynthesis in gentian flowers.

Isolation of the bHLH transcription factor gene from gentian flowers

R2R3-MYBs regulating anthocyanin biosynthesis are known to form complexes with bHLH; therefore, we also attempted to isolate *bHLH* homologs from gentian flowers. Using degenerate PCR and cDNA library screening strategies, a single candidate for the *bHLH* gene, termed *GtbHLH1*, was isolated. *GtbHLH1* comprises 2,936 bp encoding a protein of 661 amino acids starting at 354 bp from the transcription initiation site and also has two small open reading frames (ORFs; five and two amino acid residues, respectively) within the 5′-untranslated region (Supplementary Fig. S2). The deduced amino acid sequence of *GtbHLH1* exhibited 51.3, 19.7, 36.6, 17.6, 35.7 and 26.1% identities with that of petunia *AN1* and *JAF13*, Arabidopsis *TT8*, maize *Lc* and *In1*, and snapdragon *delila*, respectively. The phylogenetic tree of bHLH proteins that regulate anthocyanin biosynthesis reveals two clades, and GtbHLH1 was categorized into the same clade as petunia AN1 and Arabidopsis TT8 (Fig. 2A). The full-length genomic sequence containing the ORF was not amplified by PCR; therefore, the putative genomic structure of the *GtbHLH1* gene was deduced by assembling four overlapping PCR fragments (Fig. 2B). The *GtbHLH1* gene is >12 kb in length, which is longer than *bHLH* genes from other plants. By comparison between the genomic and cDNA sequences, the *GtbHLH1* gene was determined to have eight introns and the bHLH domain was not interrupted by an intron, which is remarkably similar to the genomic structure of petunia *AN1*. Southern bolt analysis showed that there are multiple copies of *GtbHLH1* in the gentian genome (Supplementary Fig. S3).

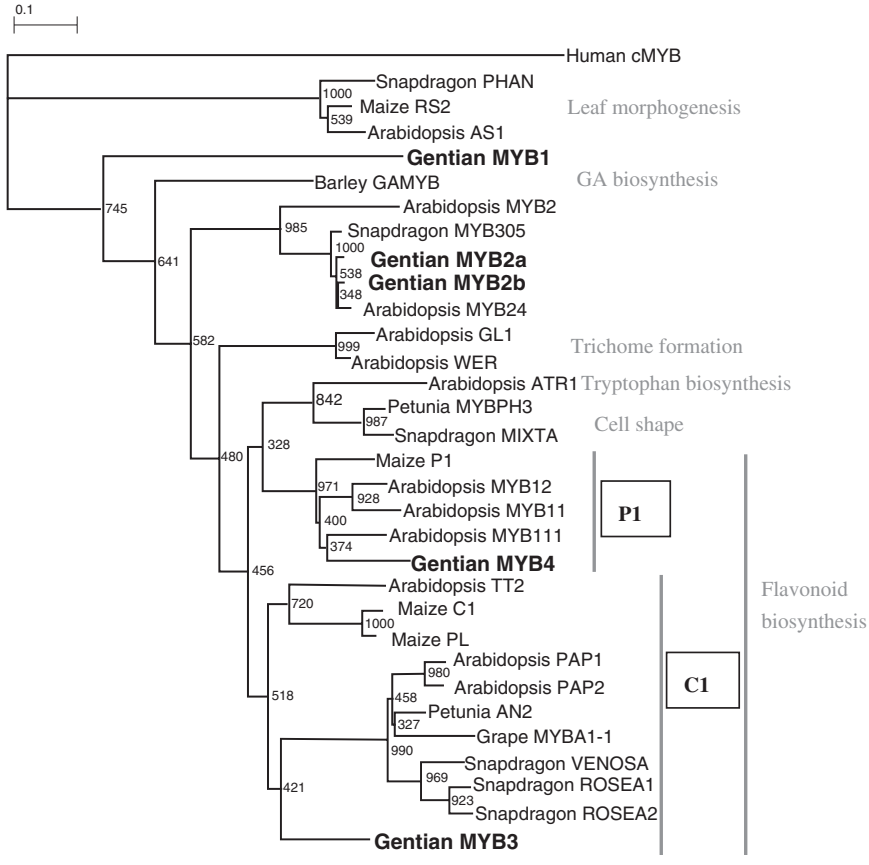

Fig. 1 Phylogenetic tree of plant R2R3-MYBs. The phylogenetic tree of the R2R3-MYB domain was generated using the CLUSTALW (Thompson et al. 1994) and TREE VIEW programs (Page 1996). Numerals next to the branches indicate bootstrap values from 1,000 replications. The bar indicates an evolutionary distance of 0.1%. Accession numbers in the GenBank/EMBL/DDBJ database are as follows. Human cMYB (M15024) was used as an out-group in this phylogenetic tree. Snapdragon PHAN (AJ005586), maize ROUGH SHEATH 2 (RS2, AF143447), Arabidopsis ASYMMETRIC LEAVES 1 (AS1, O80931), gentian MYB1 (D38165), barley GAMYB (X87690), Arabidopsis MYB2 (D14712), snapdragon MYB305 (JQ0958), gentian MYB2a (AB289443), gentian MYB2b (AB289444), Arabidopsis MYB24 (AAM63674), Arabidopsis GL1 (M79448), Arabidopsis WEREWOLF (WER, AF126399), Arabidopsis ALTERED TRYPTOPHAN REGULATION (ATR1, NM_125482), petunia MYBPH3 (Z13996), snapdragon MIXTA (X79108), maize P (U57002), Arabidopsis MYB12 (ABB03913), gentian MYB4 (AB289446), Arabidopsis TT2 (AJ299452), maize C1 (M37153), maize PL (AF015268), Arabidopsis PAP1 (AF325123), Arabidopsis PAP2 (AF325124), petunia AN2 (AF146702), grape MYBA1-1 (AB073010), snapdragon VENOSA (DQ275531), snapdragon ROSEA1 (DQ275529), snapdragon ROSEA2 (DQ275530) and gentian MYB3 (AB289445).

The expression profiles of GtMYB3, GtMYB4 and GtbHLH1

To investigate the temporal and spatial expression of *GtMYB3*, *GtMYB4* and *GtbHLH1* in gentian plants, Northern blot analysis was performed using total RNAs isolated from petals at four developmental stages, plus leaves and stems, using probes derived from each corresponding gene (Fig. 3). Accumulation of *GtMYB3* and *GtbHLH1* transcripts was detected strongly just before the anthesis stage at the beginning of flower pigmentation, but not in young unpigmented bud stages. These expression profiles of *GtMYB3* and *GtbHLH1* coincided well with the accumulation of gentiodelphin and that of genes encoding its biosynthetic enzymes, including *F3H*, *F3′5′H*, *DFR*, *ANS*, *3GT*, *5GT* and *5AT* genes (Nakatsuka et al. 2005a, Nakatsuka et al. 2008). Conversely, *GtMYB4* transcripts were detected at early flower development stages without pigmentation, but disappeared at later pigmented flower stages. This expression profile of *GtMYB4* coincided well with the accumulation profiles of flavones that exist in abundance in young petals (Nakatsuka et al. 2005a). No transcripts of *GtMYB3*, *GtMYB4* and *GtbHLH1* genes were detected in vegetative organs such as leaves and stems (Fig. 3).

Protein–protein interaction between GtMYBs and GtbHLH1

To investigate whether GtbHLH1 interacts with GtMYB3 and GtMYB4, we employed a GAL4-based yeast two-hybrid system. Since our preliminary experiment

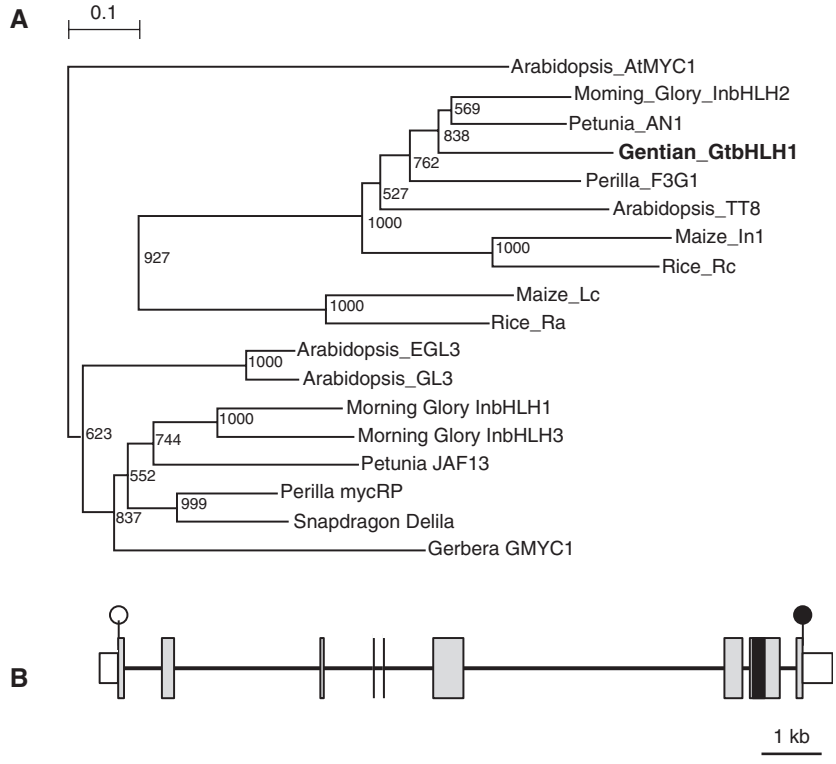

Fig. 2 Phylogenetic tree of plant bHLHs involved in anthocyanin biosynthesis. (A) Phylogenetic tree among anthocyanin biosynthesis-related bHLHs. The phylogenetic tree of the bHLH protein was generated using the CLUSTALW (Thompson et al. 1994) and TREE VIEW programs (Page 1996). Numerals next to the branches indicate bootstrap values from 1,000 replications. The bar indicates an evolutionary distance of 0.1%. Accession numbers in the GenBank/EMBL/DDBJ database are as follows. Arabidopsis MYC1 (D83511), morning glory InbHLH2 (AB232775), petunia ANTHOCYANIN1 (AN1, AF260919), gentian GtbHLH1 (this study), perilla F3G1 (AB103172), Arabidopsis TRANSPARENT TESTA 8 (TT8, NM_117050), maize IN1 (U57899), rice Rc (DQ204735), maize Lc (NM_001111869), rice Ra (U39860), Arabidopsis ENHANCER OF GLABRA3 (EGL3, NM_105042), Arabidopsis GLABRA3 (GL3, NM_148067), morning glory InbHLH1 (AB232774), morning glory InbHLH3 (AB232776), petunia JAF13 (AF020545), perilla mycRP (AB024050), snapdragon delila (M84913) and gerbera GMYC1(AJ007709). (B) Genomic structure of the *GtbHLH1* gene. Exons are indicated by boxes, and the coding regions are indicated by gray-filled boxes. The region of the bHLH domain is shown by black boxes. Open and filled circles indicate the start and stop codons, respectively.

showed that GAL4 DNA-binding domain-fused GtMYBs led to false-positive results, the interaction study was carried out with DNA-binding domain-fused GtbHLH1 and activation domain-fused GtMYBs. As shown in Fig. 4, only the yeast harboring the combination of GtMYB3 and GtbHLH1 survived on quadruple dropout medium supplemented with 3-amino-1,2,4-triazole (3-AT), which is a competitive inhibitor of the His3 protein. Although GtbHLH1 interacted with GtMYB3, GtbHLH1 did not seem to form a homodimer. Therefore, GtMYB3 and GtbHLH1 would form a heterodimer by protein–protein interaction.

Ability of GtMYB3 and GtbHLH1 to activate flavonoid biosynthetic promoters determined by transient expression assays

Temporal expression and yeast two-hybrid analyses showed that *GtMYB3* and *GtbHLH1* would be responsible for regulation of anthocyanin biosynthesis in gentian flowers. To gain an insight into the flavonoid biosynthesis activation ability of *GtMYB3* and *GtbHLH1*, transient expression assays were performed by particle bombardment in tobacco BY2 suspension cells. We chose the promoters of *GtCHS* (encoding an enzyme common to flavone and anthocyanin biosynthesis), *GtF3′5′H* and *Gt5AT* (encoding enzymes of anthocyanin biosynthesis) (Fig. 5A). The *GtCHS* promoter has several MYB-related *cis*-elements within an approximately 500 bp region upstream of the transcription start site. In contrast, *GtF3′5′H* and *Gt5AT* promoters, which were newly isolated in this study, contain some vertebrate MYB domains >500 bp upstream of the transcription start site. The results of the transient expression assays are shown in Fig. 5B. Neither *GtMYB3* nor *GtbHLH1* alone could enhance any of the tested promoter activities. However, the co-bombardment of *GtMYB3* and

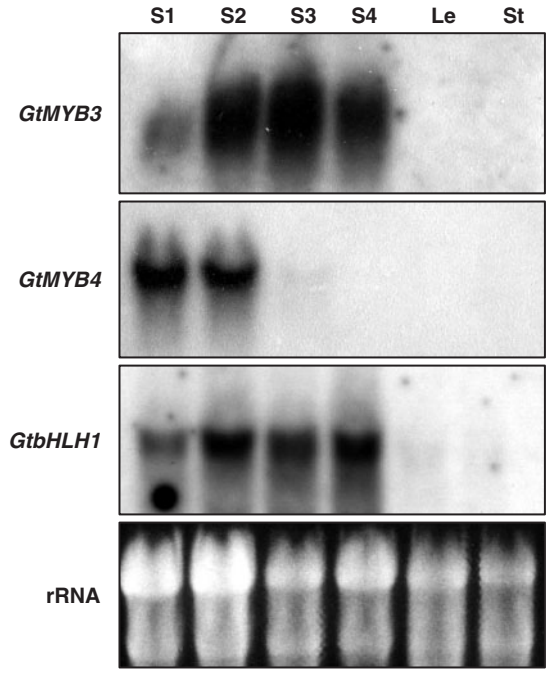

Fig. 3 Expression analysis of *GtMYB3*, *GtMYB4* and *GtbHLH1* genes. Northern blot analysis was performed using total RNA isolated from petal samples at four different flower developmental stages (S1–S4) as defined by Nakatsuka et al. (2005a), and leaf (Le) and stem samples (St). The RNAs, blotted onto nylon membrane, were hybridized with digoxigenin-labeled *GtMYB3*, *GtMYB4* or *GtbHLH1* probes as described in Materials and Methods. Ethidium bromide-stained rRNA bands are shown as a control.

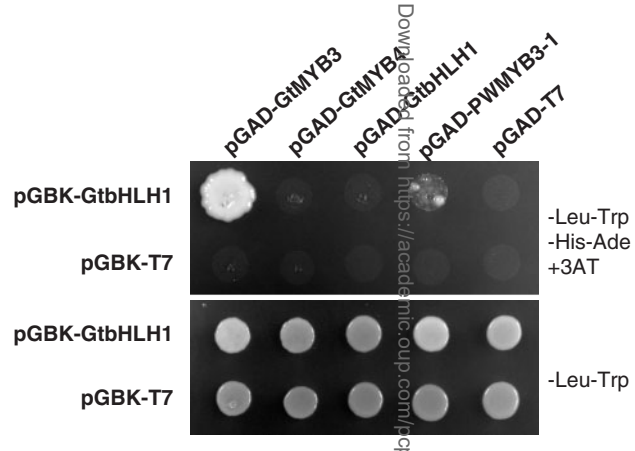

Fig. 4 Yeast two-hybrid analysis to examine the protein–protein interaction between GtMYBs and GtbHLH1. GtbHLH1 protein was fused to the GAL4 DNA-binding domain (BD) and assayed for its ability to bind the MYBs and GtbHLH1 fused to the GAL4 activation domain (AD). Interaction is shown by yeast growth on quadruple dropout medium supplemented with 15 mM 3-AT. GtMYB3 is from blue-flowered gentian and PWMYB3-1 is from the white-flowered gentian cv. 'Polarno White'.

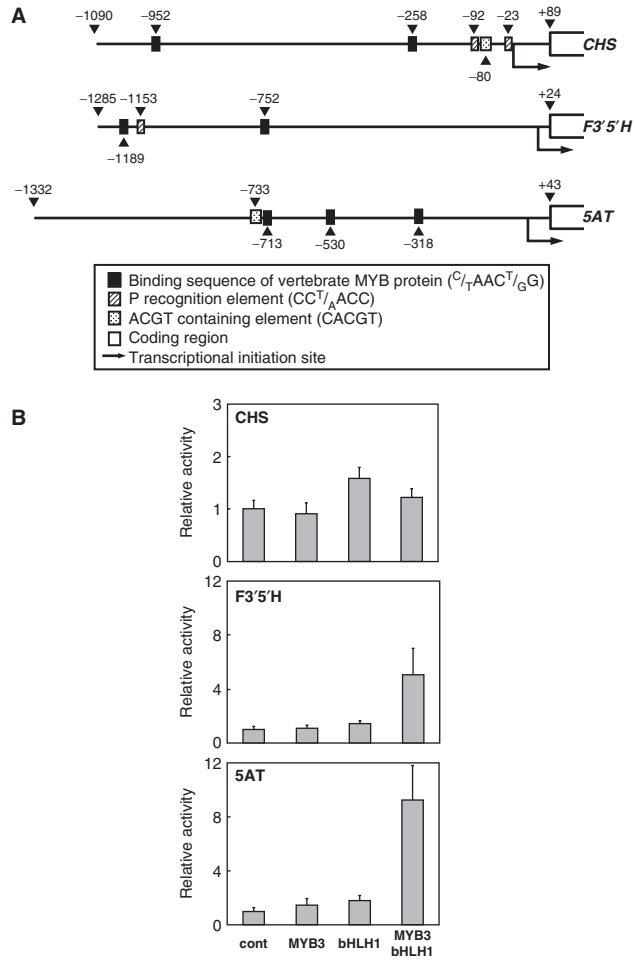

Fig. 5 Transient expression analysis of GtMYB3 and GtbHLH1 activities by particle bombardment. (A) Structures of *GtCHS*, *GtF3′5′H* and *Gt5AT* promoter. MYB recognition elements, vertebrate MYB (Urao et al. 1993), P-recognition element (Grotewold et al. 1994) and an ACGT-containing element (Hartmann et al. 1998), are shown by filled, striped and dotted boxes, respectively. The positions upstream of transcriptional initiation (arrow) are indicated as numerals above the filled triangles. The coding region is indicated as an open box. The sequence regions of −1,090 to +89, −1,285 to +24 and −1,332 to +43 as *GtCHS*, *GtF3′5′H* and *Gt5AT* promoters, respectively, were used in the transient expression assays. (B) Effect of GtMYB3 and GtbHLH1 on promoter activities of three flavonoid biosynthetic genes. Transient expression assays were performed by co-bombardment of the reporter and effector plasmid DNA into tobacco BY2 cells. GtCHSpro–GUS, GtF3′5′Hpro–GUS or Gt5ATpro–GUS were used as reporters, and p35Spro-GtMYB3, p35Spro-GtbHLH1 or pUC18 (negative control) were used as the effector. p35Spro-LUC was also used as a transformation control. After 24 h bombardment, both GUS and LUC activities were measured. The promoter activation activities of each transcription factor are indicated as relative values compared with that of a negative control, pUC18. Data indicate average value ± SD of 3–4 independent shots.

GtbHLH1 increased the promoter activities of the anthocyanin biosynthetic genes, *GtF3′5′H* and *Gt5AT*, by 5.1- and 9.3-fold respectively. In contrast, the combination did not change the promoter activity of *GtCHS*, which encodes an enzyme common to both flavone and anthocyanin biosynthesis. Therefore, we speculated that the heterodimer between *GtMYB3* and *GtbHLH1* specifically regulates the expression of anthocyanin biosynthetic genes in gentian petals.

Characterization of the GtMYB3 gene in white-flowered gentian cultivars

Our previous research demonstrated that a white-flowered gentian cultivar 'Polarno White' was an anthocyanin-defective mutant, which had decreased expression of some late flavonoid biosynthetic genes in comparison with blue-flowered gentian cultivars (Nakatsuka et al. 2005b). Therefore, we hypothesized that this white-flowered gentian would result from the mutation of certain transcription factor(s) regulating anthocyanin biosynthesis in their petals. Northern blot analysis showed that the expression of the *GtMYB3* gene of this white-flowered cultivar was at the same level as that of blue-flowered cultivars, but the expression of *GtbHLH1* was decreased slightly (Fig. 6A). However, careful observation of the size of the transcripts indicated that the *GtMYB3* transcripts from cv. 'Polarno White' were slightly longer than those from blue-flowered gentian (Fig. 6A). Moreover, reverse transcription–PCR (RT–PCR) analysis of the *GtMYB3* ORF amplified 1.2 and 1.0 kb fragments in cv. 'Polarno White' compared with 0.9 kb of normal *GtMYB3* from blue-flowered gentian (data not shown). To reveal the origins of these two abnormal sized transcripts, we amplified the genomic sequences of *GtMYB3* and obtained about 1.4 and 7.0 kb of amplified fragments. Sequence analysis showed that *GtMYB3* of cv. 'Polarno White' had two *GtMYB3* alleles, a 274 bp inserted fragment in the second exon (*myb3-1*) and an approximately 6 kb inserted fragment within an intron region (*myb3-2*) (Fig. 6B). The 274 bp inserted fragment had the features of a *hAT* transposable element, containing 8 bp of target site duplication (TSD; GGTGGACC) and 15 bp of incomplete terminal inverted repeats (TIRs; 5′- CAG$^{G}/_{T}$GGCGGACC-CAG-3′), termed *dTgt1*. The 6 kb inserted fragment does not have any characterized motifs such as transposable elements (as determined by database analysis). *myb3-1* transcribed 1.2 kb of *GtMYB3* mRNA including the 274 bp transposable element, while the *myb3-2* allele transcribed 1.0 kb of *GtMYB3* mRNA by having a new splicing acceptor site within the 6 kb inserted fragment (Fig. 6C). Both *GtMYB3* transcripts were abnormal mRNAs inducing nonsense mutations, but the *myb3-1* allele still encoded a GtMYB3 protein, albeit with a slightly truncated C-terminus (termed PWMYB3-1, Fig. 6C). A yeast two-hybrid assay showed that PWMYB3-1 interacts slightly with the GtbHLH1 protein (Fig. 4B). However, PWMYB3-1, either alone or together with GtbHLH1, could not enhance F3′5′H promoter activity in a transient expression assay (data not shown).

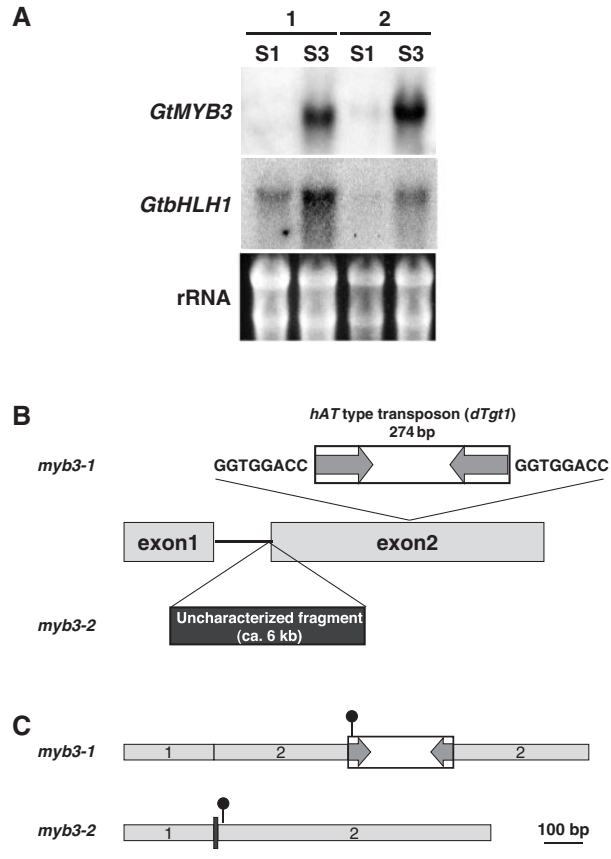

Fig. 6 Identification of mutation of white-flowered gentian cv. 'Polarno White'. (A) Expression of *GtMYB3* and *GtbHLH1* genes in white-flowered gentian cv. 'Polarno White'. Total RNA isolated from the petals at two different flower developmental stages (S1 and S3) of blue-flowered gentian cv. 'Maciry' (1) and white-flowered gentian cv. 'Polarno White' (2) were subjected to Northern blot analysis as described in Fig. 3. Ethidium bromide-stained rRNA bands are shown as a control. (B) Schematic representation of the genomic structure of the *GtMYB3* gene in cv. 'Polarno White'. *GtMYB3* from the blue-flowered cv, 'Maciry' is 1.1 kb in length, consisting of two exons and one intron. The *GtMYB3* genomic structure of the white-flowered gentian cv. 'Polarno-White' had two alleles, *myb3-1* and *myb3-2*. *myb3-1* is 1.4 kb in length, containing 274 bp of *dTgt1* transposable element (*hAT* family) within the second exon of *GtMYB3*. *myb3-2* is 7 kb in length, containing approximately 6 kb of uncharacterized fragment within the intron region. (C) Schematic representation of *GtMYB3* transcripts in cv. 'Polarno White'. *GtMYB3* transcripts from blue-flowered gentian are 0.9 kb in length. The *myb3-1* allele from cv. 'Polarno White' transcribed 1.2 kb of abnormal *GtMYB3* including the transposable element *dTgt1*. The *myb3-2* allele transcribed 1.0 kb of abnormal *GtMYB3*, derived from mis-splicing within the approximately 6.0 kb inserted fragment. Filled circles indicate the ectopic stop codons resulting from insertion mutations.

Moreover, six other white-flowered gentian cultivars also had several nonsense mutations by insertion of a single nucleotide or transposon-like elements in the *GtMYB3* gene (Supplementary Fig. S4), indicating that the loss of functional *GtMYB3* expression is responsible for the deficiency of anthocyanin development in white-flowered gentian, although the exact expression and sequence analyses should be performed for each cultivar in future studies. No mutation of *GtbHLH1* was found in any of the seven white-flowered gentian cultivars tested in this study. These results strongly suggested that *GtMYB3* regulates anthocyanin (gentiodelphin) biosynthesis and that mutations of the *GtMYB3* gene are involved in white coloration in gentian petals.

Discussion

Our previous study indicated that gentian flower pigmentation was temporally and spatially controlled by the level of transcription of flavonoid biosynthetic genes (Nakatsuka et al. 2005a). However, no transcription factors regulating flavonoid biosynthesis have been identified in gentian plants to date. In model plants such as Arabidopsis, maize, perilla, snapdragon and petunia, both *R2R3-MYB* and *bHLH* genes are known to be responsible for anthocyanin biosynthesis as transcription factors (Koes et al. 2005, Quattrocchio et al. 2006). Degenerate PCR technology successfully identified 26 *R2R3-MYB* homologs and one *bHLH* homolog from gentian flowers. Since bHLH proteins have low similarity among orthologs in comparison with R2R3-MYB proteins, it was thought that the degenerate primers designed in this study could not amplify the wide range of *bHLH* genes. Of the isolated R2R3-MYBs from gentian flowers, both GtMYB3 and GtMYB4 were categorized into the R2R3-MYB clade regulating flavonoid biosynthesis by phylogenetic analysis (Fig. 1). GtMYB3 exhibited remarkable similarity to petunia AN2, Arabidopsis PAP1 and TT2, and maize C1, which are responsible for anthocyanin and proanthocyanin biosyntheses (Fig. 1, Supplementary Fig. S1); therefore, GtMYB3 was chosen for further study. The functional analysis of GtMYB4 will be performed in future studies. GtMYB4 shows identities with AtMYB11, AtMYB12, AtMYB111, maize P1 and sorghum Y1, which are considered to regulate flavonol or phlobaphene biosynthesis. In the case of gentian, *GtMYB4* might be responsible for early flavonoid biosynthesis, especially flavones, considering its expression profile.

The phylogenetic tree of bHLH proteins related to anthocyanin biosynthesis fell into two different clades (Spelt et al. 2000). GtbHLH1 exhibited high identity with that of petunia AN1, controlling anthocyanin biosynthesis and vacuole pH in petunia flowers (Spelt et al. 2000, Spelt et al. 2002) (Fig. 2A). The *GtbHLH1* gene has eight introns, as does petunia *AN1* (Spelt et al. 2000) (Fig. 2B). Although the bHLH domains of maize *In1* and petunia *JAF13* are separated by an intron, the bHLH domain of *GtbHLH1* is encoded within a single exon, just like petunia *AN1* (Spelt et al. 2000), morning glory *InbHLH2* (Park et al. 2007) and Arabidopsis *TT8* (Nesi et al. 2000). Therefore, phylogenetic and genomic structure analyses suggested that *GtbHLH1* evolved from a common ancestral gene corresponding to petunia *AN1* and might regulate anthocyanin biosynthesis in gentian flowers. Maize, petunia and morning glory also have at least two bHLH orthologs belonging to each clade (Spelt et al. 2000, Park et al. 2007). Southern blot analysis showed that *GtbHLH1* existed in multiple copies in the gentian genome (Supplementary Fig. S3). However, no homologs corresponding to Arabidopsis *GL3* and *EGL3*, snapdragon *delila*, morning glory *InbHLH1* and petunia *JAF13* were isolated from gentian flowers in this study. Thus, it will be necessary to carry out further studies to identify other *bHLH* homologs in gentian flowers.

The accumulation of *GtMYB3* and *GtbHLH1* transcripts increased according to the degree of flower pigmentation in gentian, and nearly corresponded to the profiles of gentiodelphin accumulation except that gene expression was slightly earlier than pigment accumulation (Fig. 3 and Nakatsuka et al. 2005a). It may be reasonable that the transcriptions precede the onset of anthocyanin accumulation. Gentiodelphin biosynthesis was shown to be controlled by the expression levels of anthocyanin biosynthetic genes, including *F3H*, *F3′5′H*, *DFR*, *ANS*, *3GT*, *5GT* and *5AT* (Nakatsuka et al. 2005a, Nakatsuka et al. 2008). The expression profiles of *GtMYB3* and *GtbHLH1* genes were similar to those of the anthocyanin biosynthetic genes. Therefore, the expression analysis data together with phylogenetic analysis implied that *GtMYB3* and *GtbHLH1* might regulate the expression of anthocyanin biosynthetic genes in gentian flowers.

Yeast two-hybrid analysis showed that GtbHLH1 interacts with GtMYB3 but not GtMYB4 (Fig. 4). Arabidopsis bHLH proteins such as TT8 and EGL also interact with R2R3-MYBs of subgroup 5, containing PAP1, PAP2, AtMYB113 and AtMYB114 (Zimmermann et al. 2004). The interaction between the maize C1 and B proteins requires amino acids within 1–117 of C1 and 1–244 of B (Goff et al. 1992). Although the interaction domain of GtbHLH1 and GtMYB3 has not been identified in this study, it could be speculated that they are similar to those of maize C1 and B proteins.

Transient expression analysis showed that co-expression of *GtMYB3* and *GtbHLH1* could enhance the promoter activities of *GtF3′5′H* and *Gt5AT*, encoding enzymes involved in anthocyanin biosynthesis, but not those of *GtCHS*, encoding an early flavonoid biosynthetic enzyme (Fig. 5). *GtMYB3* or *GtbHLH1* alone could not enhance

any tested promoter activity. Maize C1 and petunia AN2 were reported to require direct interaction with bHLH, R and AN1, respectively, for activation (Goff et al. 1992, Spelt et al. 2000). The interaction of GtMYB3 with GtbHLH1 also seems to be necessary for regulating anthocyanin structural genes in gentian flowers. Petunia, Arabidopsis and maize have some redundant homologs for R2R3-MYB and bHLH in their genomes. More recently, it has been reported that Arabidopsis MYBL2, a small MYB protein, acts as a negative regulator for anthocyanin biosynthesis (Dubos et al. 2008, Matsui et al. 2008). Therefore, anthocyanin biosynthesis in gentian flowers could be controlled by more complex regulation. Other genes regulating anthocyanin biosynthesis, besides *GtMYB3* and *GtbHLH1*, could be present in the gentian genome, and these genes must be exhaustively cloned in future studies. Similarly, WDR proteins remain to be isolated, and combinational studies using these three protein families should be performed to achieve full understanding of the regulation of flavonoid biosynthesis in gentian flowers.

Flower color mutants are useful for investigating genes involved in the flavonoid biosynthetic pathway. Our previous study showed that white-flowered gentians were divided into two different mutant types, deficiency of the *ANS* gene and mutations of anthocyanin biosynthetic regulatory factor(s) (Nakatsuka et al. 2005b). The flowers of cv. 'Polarno White' expressed the abnormal *GtMYB3* transcripts, harboring insertions of a DNA transposable-like element or alternative splicing (Fig. 6). Some other white-flowered gentian cultivars also had insertions of single nucleotides or DNA transposable-like elements in their *GtMYB3* gene (Supplementary Fig. S4). These results provided direct evidence that *GtMYB3* plays an important role in regulating anthocyanin biosynthesis in gentian flowers. White-flowered gentian cv. 'Polarno White' has normal genomic structures for all anthocyanin biosynthetic genes, and the pigmentation of anthocyanin in their petals and leaves can be induced when exposed to environmental stress (Nakatsuka et al. 2005b). Northern blot analysis showed that the expression of *GtbHLH1* in the petals of cv. 'Polarno White' was decreased compared with blue-flowered gentian. In Arabidopsis, the MYB–bHLH–WDR complex can also regulate the expression of *TT8*, encoding a bHLH protein, in a positive feedback loop that ensures a strong accumulation of proanthocyanin in the endothelium (Baudry et al. 2006). Therefore, it is likely that functional mutation of *GtMYB3* in cv. 'Polarno White' might induce the reduced accumulation of *GtbHLH1* transcripts. Although PWMYB3-1 (truncated GtMYB3 encoded by the *myb3-1* gene) from cv. 'Polarno White' could not activate expression of anthocyanin biosynthetic genes in a transient expression assay (data not shown), PWMYB3-1 could interact weakly with GtbHLH1 in a yeast two-hybrid analysis (Fig. 4). Since the N-terminal 189 amino acids of PWMYB3-1 were translated normally, the interaction domain with GtbHLH1 might exist in this N-terminal region of PWMYB3-1. The *dTgt1* inserted in *myb3-1* of cv. 'Polarno White' was a novel type *hAT* transposable element in gentian, compared with the sequences of the TSD and TIR of *dTgs1* inserted in the *F3′5′H* gene (Nakatsuka et al. 2006). No revertant blue flowers have been obtained in cv. 'Polarno White', suggesting that *dTgs1* is not active under normal cultivation conditions. Complementation of the white-flowered mutant phenotype with *GtMYB3* would provide the final confirmation; therefore, we are now attempting to produce *GtMYB3*-overexpressing white-flowered gentians.

In conclusion, this study demonstrated that two newly identified transcription factors, GtMYB3 and GtbHLH1, are definitely involved in the regulation of anthocyanin biosynthesis in gentian flowers, and that white-flowered gentian mutants were caused by the functional deficiency of GtMYB3. Further analyses using transgenic plants are now in progress to elucidate the molecular mechanisms of gentian petal pigmentation. In addition, the transcription factor genes isolated here might be useful for basic studies of flavonoid biosynthesis and for modification of flower colors in gentian plants by genetic engineering.

Materials and Methods

Plant materials

Blue-flowered gentian (*G. triflora*) cv. 'Maciry' and white-flowered interspecific gentian (*G. triflora* × *G. scabra*) cv. 'Polarno White', growing in the fields of the Iwate Agriculture Research Center (Iwate, Japan), were used in this study. Other white-flowered gentian cultivars were purchased or provided by the Iwate Agriculture Research Center. Their petal samples at four different stages as defined by Nakatsuka et al. (2005a) were collected and stored –80°C until used.

Isolation of R2R3-MYB homologs from gentian petals

Total RNA was isolated from petals at the flower developmental stages 1 (S1) to 3 (S3) as defined by Nakatsuka et al. (2005a). cDNA was synthesized using a Takara RNA PCR (AMV) kit version 2.1 (TAKARA BIO INC., Tokyo, Japan). Degenerate primers were designed from the conserved DNA-binding domain of R2R3-MYBs controlling flavonoid biosynthesis in other plant species and are shown in Supplementary Table S1. The other degenerate primers as described by Rabinowicz et al. (1999) were also used in this study. A 25 µl aliquot of the PCR mixture contained 1 µl of first-strand cDNA, 1 × *Ex* buffer, 200 µM dNTPs, 5 µM of each primer and 1.25 U of *Ex Taq* polymerase (TAKARA BIO INC.). Reaction conditions consisted of pre-heating at 94°C for 90 s, 40 cycles at 95°C for 20 s, 50°C for 40 s and 72°C for 1 min, and an extension at 72°C for 10 min. The amplified fragments were subcloned into the PCR4-TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) and subjected to sequence analysis using a Big-Dye terminal cycle sequencing kit version 1.1 and an ABI PRISM 3100 DNA sequencer (Applied Biosystems Japan, Tokyo, Japan). Nucleotide sequences were translated to the respective

deduced amino acid sequences using DNASIS version 3.6 (HITACHI, Tokyo, Japan) and compared using the BLAST network service from the National Center for Biotechnology Information (NCBI). A phylogenetic tree was produced using CLUSTALW (Thompson et al. 1994) and TREEVIEW version 1.6.6 programs (Page 1996).

To obtain full-length cDNA of each *GtMYB*, 3′- and 5′-RACE technology was performed using a GeneRacer kit (Invitrogen) with the primers for 3′- and 5′-RACE shown in Supplementary Table S1. The amplified fragments were subcloned and subjected to sequence analysis as described above. The full-length cDNA sequence of the *GtMYB* genes was assembled into the 3′- and 5′-RACE sequences using the GENETYX-MAC software version 12.0 (GENETYX, Tokyo, Japan).

Isolation of bHLH homologs from gentian petals

Degenerate primers for isolation of *bHLH* were designed from conserved amino acid sequences among *delila* (*A. majus*, accession No. 84913), *GMYC1* (*Gerbera hybrida*, AJ007709), *MYC-RP* (*Perilla frutescens*, AB024050), *AN1* (*P. hybrida*, AF260919) and *JAF13* (*P. hybrida*, AF020545), and are shown in Supplementary Table S1. The PCR mixture and conditions were as described above. An approximately 360 bp amplified fragment for *bHLH* was subcloned into the pCR4-TOPO TA cloning vector (Invitrogen), and was labeled and used to screen the flower cDNA library from *G. triflora* cv. 'Maciry' (Kobayashi et al. 1998) using the ECL Direct Nucleic Acid Labeling and detection System (GE Healthcare, Uppsala, Sweden) as described by Nakatsuka et al. (2005a). Screening a 4×10^6 p.f.u. cDNA library, several positive clones were identified, isolated by in vivo excision and then sequenced. To obtain the full-length cDNA of *GtbHLH1*, 5′-RACE was performed using a primer as shown in Supplementary Table S1.

Identification of the genomic structure of GtMYB3 and GtbHLH1 genes

Genomic DNA was isolated from 1 g of young leaves of gentian plants using Nucleon PhytoPure (GE Healthcare). Genomic *GtMYB3* and *GtbHLH1* genes were amplified using some of the primers shown in Supplementary Table S1. The PCR for *GtMYB3* was performed as described above. For genomic *GtbHLH1*, the reaction mixture, in a total volume of 25 µl, contained 200 ng of genomic DNA from gentian petals, 200 µM dNTPs, 0.4 µM of each primer, 1.25 U of PrimeSTAR GXL DNA Polymerase (TAKARA BIO INC.) and 1× PrimeSTAR GXL Buffer. The reaction conditions were 30 cycles at 98°C for 10 s, 60°C for 15 s and 68°C for 10 min. The reaction mixture was purified using a MicroSpin S-400HR column (GE Healthcare) and then subcloned into the pCR4-TOPO TA cloning vector (Invitrogen). Sequence analysis was performed as described above. A full-length genomic sequence of *GtbHLH1* was assembled from the fragments using ATGC software (GENETYX).

Expression analysis of GtMYBs and GtbHLH1 in gentian

To investigate the temporal and spatial expressions of the *GtMYBs* and *GtbHLH1* genes in the *G. triflora* blue-flowered gentian cv. 'Maciry' and white-flowered gentian cv. 'Polarno White', Northern blot analysis was performed using total RNA isolated from petals at the four different flower developmental stages defined as in Nakatsuka et al. (2005a), and from mature leaves and stems. A 5 µg aliquot of total RNA was separated on a 1.25% MOPS-agarose gel and then transferred to Hybond-N+ membranes (GE Healthcare). Probes for the flavonoid biosynthesis genes were prepared with a PCR-DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany) using each primer set as described in Supplementary Table S1. The membranes were hybridized and detected as described by Nakatsuka et al. (2005a).

Yeast two-hybrid analysis

In the yeast two-hybrid assay, the Matchmaker Two-Hybrid System 3 (Clontech, Mountain View, CA, USA) was employed to investigate the protein interactions between GtbHLH1 and GtMYBs. The ORF sequences of *GtMYB3*, *GtMYB4* and *GtbHLH1* were amplified using primer sets as shown in Supplementary Table S1, and then cloned into the pGAD-T7 and pGBK-T7 vectors (Clontech). DNA encoding PWMYB3-1, which is a C-terminally truncated GtMYB3 found in cv. 'Polarno White' transcripts, was also used. All constructs were transformed into *Saccharomyces cerevisiae* strain AH109 (Clontech) using the *S.c.* easyComp transformation kit (Invitrogen). Yeast transformants were grown on selective medium without leucine (–LEU) and tryptophan (–TRP) at 30°C for 3 d and tested on selective medium without leucine, tryptophan, histidine and adenine, and supplemented with 3-AT, which is a competitive inhibitor of the His3 protein.

Isolation of 5′-upstream regions of F3′5′H and 5AT genes in gentian

The 5′-upstream regions of the gentian *F3′5′H* and *5AT* genes were identified using inverse PCR. A 1 µg aliquot of *G. triflora* cv. 'Maciry' genomic DNA was digested by each restriction enzyme and self-ligated by a Takara ligation Kit version 3.0 (TAKARA BIO INC.). Inverse PCR was performed in a 25 µl reaction mixture containing 100 ng of ligated genomic DNA, 1 × *LA* buffer, 2.5 mM $MgCl_2$, 400 µM dNTPs, 0.2 µM of each primer, and 1.25 U of *LA Taq* polymerase (TAKARA BIO INC.). The primer sets used are described in Supplementary Table S1. Reaction conditions consisted of pre-heating at 94°C for 90 s, 35 cycles at 95°C for 20 s, 60°C for 40 s and 72°C for 3 min, and an extension at 72°C for 10 min. Amplified fragments of about 2.0 kb for *GtF3′5′H* and 2.1 kb for *Gt5AT* were subcloned and sequenced as described above. Putative transcriptional initiation points of *GtF3′5′H* and *Gt5AT* genes were also determined by 5′-RACE technology as described above.

Vector construction for transient expression assay

The reporter vectors were constructed to contain the β-glucuronidase (GUS) gene under the control of the gentian *CHS*, *F3′5′H* or *5AT* promoters and a nopaline synthase (NOS) terminator from *Agrobacterium tumefaciens*. The primers for cloning of the *GtF3′5′H* and *Gt5AT* promoters were designed from sequence information obtained by inverse PCR and are shown in Supplementary Table S1. The GtF3′5′Hpro–GUS and Gt5ATpro–GUS vectors were constructed to substitute the promoter region [cauliflower mosaic virus (CaMV) 35S promoter] of the modified pBI221 with each 5′-upstream fragment. GtCHSpro–GUS constructed by Kobayashi et al. (1998) was also used.

The *GtMYB3* and *GtbHLH1* ORF fragments were amplified using the primer set as shown in Supplementary Table S1 and subcloned into the PCR4-TOPO TA cloning vector (Invitrogen). 35Spro-GtMYB3 and 35Spro-GtbHLH1 vectors were constructed to replace the *GUS* gene of pBI221 with each ORF.

Transient expression assay using tobacco BY2 cells

To evaluate whether *GtMYB3 and GtbHLH1* were responsible for the regulation of anthocyanin biosynthesis in gentian flowers, transient expression assays were performed using tobacco suspension-cultured BY2 cells and the particle bombardment system PDS-1000/He (Bio-Rad, Hercules, CA, USA). A 0.5 mg aliquot of gold particles was precipitated together with the three plasmids, containing 2.5 μg of reporter vector (GtCHSpro–GUS, GtF3′5′Hpro–GUS or Gt5ATpro–GUS), 1.25 μg of effector vector (35Spro-GtMYB3 and/or 35Spro-GtbHLH1 or pUC18) and 0.6 μg of 35Spro-LUC as transformation control per shot, and were transformed into 4-day-old BY2 cells according to a previously described protocol (Nakatsuka et al, 2005b). After 24 h of bombardment, GUS activities were measured using 4-methyl umbelliferone glucuronide (4-MUG) as a substrate (as described by Jefferson et al. 1987). In addition, LUC activities were also measured using a PicaGene Kit (Toyo Ink, Tokyo, Japan) and Luminescencer JNR II (ATTO, Tokyo, Japan) according to the manufacturer's instructions. Promoter activities were indicated as the relative GUS activity compared with the value of the vector control after normalization by LUC values. All experiments were performed in triplicate. At least three independent shots were done for each plasmid combination to demonstrate reproducibility.

Supplementary data

Supplementary data are available at PCP online.

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