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Two different mutations are involved in the formation of white-flowered gentian plants☆

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Abstract

Japanese cultivated gentian plants have naturally blue flowers, but some white-flowered cultivars are being bred through the utilization of spontaneous mutants. To determine the molecular basis of white coloration in gentian flowers, we compared two white-flowered cultivars Homoi and Polano White to a blue-flowered cultivar Maciry using biochemical and molecular approaches. High performance liquid chromatography (HPLC) analyses showed that flavone levels in cv. Polano White were only about one-half the amounts measured in the other two cultivars, while anthocyanins were absent in the two white-flowered cultivars compared to cv. Maciry in which high levels accumulated. Northern blot analysis of 10 flavonoid biosynthetic structural genes, previously reported to be temporally regulated in cv. Maciry [1] showed that cv. Homoi lacked transcripts for the anthocyanidin synthase (*ANS*) gene while cv. Polano White had decreased expressions for *ANS* as well as for chalcone synthase (*CHS*), flavanone 3-hydroxylase (*F3H*), flavonoid 3′,5′-hydroxylase (*F3′,5′H*), dihydroflavonol 4-reductase (*DFR*), UDP-glucose:flavonoid 3-glucosyltransferase (*3GT*) and anthocyanin 5-aromatic acyltransferase (*5AT*). Southern blot analysis confirmed the deficiency of one of two *ANS* loci in cv. Homoi. Transient expression of *ANS* in flower petals also strongly suggested that white flowers of cv. Homoi were derived from *ANS* mutation. Furthermore, analysis of stress-induced flower pigmentation suggested that rather than mutations in multiple structural genes being the cause, a defect in one or more regulatory factors controlling the later steps of flavonoid biosynthesis is responsible for white coloration in cv. Polano White.

Keywords: Anthocyanidin synthase; Flavonoid; Gene expression; Gentian; Mutation; White flower

1. Introduction

One of the important objectives when breeding ornamental flowers is to obtain a wide variety of flower colors. Flower coloration is the result of an accumulation of secondary metabolites, such as flavonoid, carotenoid and betalain compounds. Of these, flavonoid pigments, which are widespread in higher plants, have been studied extensively [2–4].

Flavonoids are a diverse group of phenolic compounds consisting of two aromatic rings joined by a C3 unit. In addition to their role in flower pigmentation, they are also known to be involved in attraction of pollinators, plant–microorganism interactions, protection from harmful UV irradiation, pollen tube growth and tissue pigmentation [3,4]. The biochemistry, genetics and molecular biology of flavonoids, especially with regard to anthocyanin pigmentation, have been elucidated in maize (*Zea mays*) kernels, flowers of petunia (*Petunia hybrida*) and snapdragon (*Antirrhinum majus*), and the seeds and leaves of *Arabidopsis thaliana* [2–5].

Mutations that alter flower color have long been selected and used by horticulturalists in breeding new varieties.

Abbreviations: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′,5′H, flavonoid 3′,5′-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose:flavonoid 3-glucosyltransferase; 5AT, anthocyanin 5-aromatic acyltransferase; FSII, flavone synthase II; HPLC, high performance liquid chromatography; RT-PCR, reverse transcription-polymerase chain reaction

☆ The nucleotide sequence reported in this paper has been submitted to DDBJ, EMBL and GenBank under accession number AB208689 (*GtANS*).

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Natural mutants were once selected from wild genetic resources, but nowadays, aggressive mutations are commonly introduced through artificial strategies, such as radiation irradiation and chemical mutagens [6]. Flower color mutants have also been used for characterization of structural enzymes (genes) and regulatory factors related to flavonoid biosynthesis and for isolation of transposable elements [5]. Moreover, these findings have been directly used for modification of flower color by genetic transformation technology [7].

Variation in flower color is generally the result of differences in either structural or regulatory genes involved in the flavonoid biosynthetic pathway. In most cases, visible changes are caused by differences in the amount and components of flower pigments. For example, mutations that block the early steps of flavonoid biosynthesis result in the formation of white flowers through accumulation of colorless pigments, while blockage in later steps results in formation of different colored flowers through accumulation of a particular anthocyanin. In addition, white flower phenotypes show color variation from pure white to ivory depending on which flavonoid biosynthesis step is blocked. For example, in *A. majus* [5,8,9], *Pharbitis nil* [10,11], *Dianthus caryophyllus* [12,13] and *Eustoma grandiflorum* [14], ivory flowers with an accumulation of colorless flavonoids in their petals are known to result from mutations in the genes encoding flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR) or anthocyanidin synthase (ANS) during later steps of flavonoid biosynthesis. On the other hand, pure white flowers with an accumulation of organic acids but not flavonoid compounds [15] are known to occur as a result of a *niv* mutation in *A. majus* [16] and *f* mutation in *Matthiola incana* [17,18], both of which affect chalcone synthase. Various mutants of flavonoid biosynthesis regulatory factors also result in white flower phenotypes; for example, *an1* [19] and *an2* [20] mutants in *P. hybrida*, and the *g* mutant in *M. incana* [21]. In the case of *A. majus*, combinatorial control by three alleles, *delila, Eluta* and *rosea*, is known to be involved in spatial regulation of flower pigmentation [5,9]. Japanese cultivated gentians, such as *Gentiana triflora, G. scabra* and their hybrids, are popular ornamental flowers in Japan, common as cut flowers and potted plants [23]. However, mainly due to the limited natural sources of color variation, they have a narrow range of flower coloration (original blue, pale blue, pink and white). So far, no artificial mutations, except for a dwarf phenotype introduced by *Agrobacterium rhizogenes* [24], have been expressed in gentians. Several reports have characterized flavonoid biosynthesis structural genes isolated from blue-flowered gentians [25–28] in addition to carrying out pigment analysis [29,30]. More recently, we isolated four structural genes, *F3H, ANS*, flavonoid 3′-hydroxylase (*F3′H*) and flavone synthase (*FSII*), and reported a strong correlation between their expression patterns and pigment accumulation in blue-flowered cultivars [1].

In this study, we aimed to investigate the cause of white flower coloration in gentian plants, and revealed two different mutations in two respective white-colored cultivars. One was a structural gene (*ANS*) mutation and the other a mutation of a regulatory factor controlling the expression of genes catalyzed during later steps of flavonoid biosynthesis. To the best of our knowledge, this is the first report documenting molecular analysis of flower color mutations in gentian plants.

2. Materials and methods

2.1. Plant material

Gentiana triflora cv. Maciry (blue flowers), cv. Homoi (white flowers) and *G. triflora* × *G. scabra* cv. Polano White (white flowers) were grown in the field of Iwate Agriculture Research Center. Petals at two different flower-development stages, the bud stage (S1) and just before anthesis (S3), as defined by Nakatsuka et al. [1], and leaf samples were collected from all cultivars and stored at −80 °C until use.

2.2. Extraction and analysis of petal flavonoids

Petal samples were extracted from each gentian cultivar for high performance liquid chromatography (HPLC) analysis of flavonoids. For anthocyanidin analysis, frozen petal, leaf and stem samples (250 mg) were ground in liquid nitrogen, then extracted using 1 ml EAA solution (ethanol:acetic acid:water = 10:1:9) at 4 °C overnight. For hydrolysis of anthocyanin, 4.5 ml of 3.2N HCl was added to the supernatant before incubation at 100 °C for 90 min. The hydrolysis solution was extracted with 1 ml isoamylalcohol and filtrated through a 0.22 μm filter (Millipore, Tokyo, Japan). The filtrated samples were analyzed by HPLC (D-7000 HPLC system manager, HITACHI) with a reverse phase column (J' sphere ODS M80; 4.6 mm × 150 mm, YMC) using 15% (v/v) acetonitrile and 3% (v/v) acetate acid as the solvent for 20 min at 40 °C at a flow rate of 1.0 ml/min. Anthocyanidins were quantified by monitoring the peak area of absorbance at 500 nm using respective authentic compounds as the standards. For colorless pigment analysis, flavone and flavonol in the petals were extracted and determined as described previously [1]. Flavone and flavonol were quantified by monitoring the peak area of absorbance at 330 and 360 nm, respectively.

2.3. Expression analysis of flavonoid biosynthesis structural genes

Total RNAs were isolated from petals of all three gentian cultivars at two different flower developmental stages (S1 and S3). Northern blot analysis of flavonoid biosynthesis structural genes was carried out using the DIG system (Roche) as described by Nakatsuka et al. [1].

2.4. Southern blot analysis of flavonoid biosynthesis structural genes

Total genomic DNA was isolated from 10 g leaf samples using the CTAB method as described by Murray and Thompson [31] with slight modifications according to Doyle and Doyle [32]. After digestion with either *Eco*RI or *Hin*dIII (TaKaRa, Tokyo), the genomic DNA was separated on 0.6% (w/v) agarose, gel then transferred to Nytran N membranes (Schleicher & Schuell, Germany). Southern blot analysis was performed as described by Nakatsuka et al. [1]. The *ANS* probe was prepared with a DIG-high prime DNA labeling kit (Roche Diagnostics, Germany) using the open reading frame (ORF) of Gentian *ANS* cDNA. The forward and reverse primers used for PCR amplification were 5′-AATGGGATCTCTTTTGCCTAGTAG (the initiation codon is underlined) and 5′-TCCTAATTGCCATTAGCAATATTG-3′ (the stop codon is underlined), respectively. The probes for all other structural genes were prepared and used as for northern blot analysis.

2.5. Amplification and sequence determination of the gentian genomic ANS gene

Total genomic DNAs extracted, as described above, were used for amplification of the genomic *ANS* gene using the above primer pairs. They were then subcloned into the pCR4-TOPO TA cloning vector (Invitrogen, CA) and sequenced. Sequence analyses were carried out using a Big-Dye Terminator cycle sequencing Kit (version 1.1) and the DNA sequencer model ABI PRISM 377 (Applied Biosystems Japan, Tokyo).

2.6. Construction of a recombinant plasmid

Conventional molecular biology protocols were essentially followed as described by Sambrook et al. [33]. The ORF of gentian cv. Maciry *ANS* cDNA was amplified with the above primers, then sub-cloned into the cloning vector pCR2.1 (Invitrogen). A *GtANS* fragment was removed by digestion with *Bam*HI and *Xho*I from the multi-cloning site of the cloning vector. Next, p35S-GtANS-containing *GtANS* cDNA, under control of the Cauliflower mosaic virus 35S (CaMV35S) promoter, was constructed by replacing the *gus* gene of pBI221 (BD Biosciences Clontech). A p35S-sGFP vector [34], which contains a modified *gfp* gene driven by the CaMV35S promoter, was used as the transformation control plasmid.

2.7. Complementation of ANS through transient expression in cv. Homoi petals

To investigate the *ANS* gene deficiency in cv. Homoi, a complementation experiment was performed by transient expression using particle bombardment. S3 petals of cv. Homoi were washed twice with sterilized water, then placed in the center of regeneration medium (MS basal medium containing 5 mg/l 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (TDZ) and 0.1 mg/l naphthalene acetic acid (NAA), 10 mg/l meropenum, 30 g/l sucrose and 2 g/l gellan gum; Hosokawa et al. [35]) for 2 days prior to bombardment. Particle bombardment was performed using a PDS-1000/He Biolistic Particle Delivery System (Bio-rad, CA). Two micrograms of p35S-GtANS and p35S-sGFP plasmid DNA was co-precipitated onto 0.5 mg of gold particles (1 μm in diameter) per shot according to the protocol described by Perl et al. [36] with slight modification. Petals were bombarded at a pressure of 7.5 MPa from a distance of 10 cm away from the macrocarrier holder; each sample was bombarded twice. Microscope observations were performed 3 days after bombardment under light or mercury lamp (UV-B) conditions.

3. Results

3.1. Flavonoid accumulation in the blue- and white-flowered gentian cultivars

The three gentian cultivars used in this study are shown in Fig. 1. (A) is the blue-flowered cultivar Maciry, while (B) and (C) are the white-flowered cultivars Homoi and Polano White, respectively, both of which have ivory white, not pure white, flowers. Cv. Maciry and cv. Polano White show anthocyanin accumulation in the stems, whereas cv. Homoi shows no pigments throughout.

To compare the accumulation of flavonoid compounds among the petals of blue- and white-flowered cultivars, flavonoid pigments were extracted from S4 petals and subjected to HPLC analysis (Table 1 and Fig. 1). Anthocyanin analyses showed that the blue-flowered cv. Maciry accumulated delphinidin derivatives (602.3 μg delphinidin/g fresh weight) in its petals, whereas the white-flowered cultivars accumulated undetectable levels of anthocyanin derivatives as predicted from their flower colors. Flavone derivatives, which are generally known to accumulate in the petals of gentians as colorless pigments, were also analyzed. In the petals of cv. Homoi and cv. Polano White, apigenin derivatives accumulated at a higher level than luteolin derivatives, and these flavone profiles were

Table 1
Flavonoid content in the petals of three gentian cultivars determined by HPLC analysis

Cultivar	Flower color	Flavonoid concentration (μg/g FW)		
		Anthocyanidin	Flavone	
		Delphinidin	Apigenin	Luteolin
Maciry	Blue	602.3	86.1	13.9
Homoi	Ivory white	ND[b]	74.2	27.3
Polano White	Ivory white[a]	ND	47.5	17.1

[a] This cultivar often shows pink pigmentation in its flowers and leaves as a result of enviromental stress.
[b] ND, not detected.

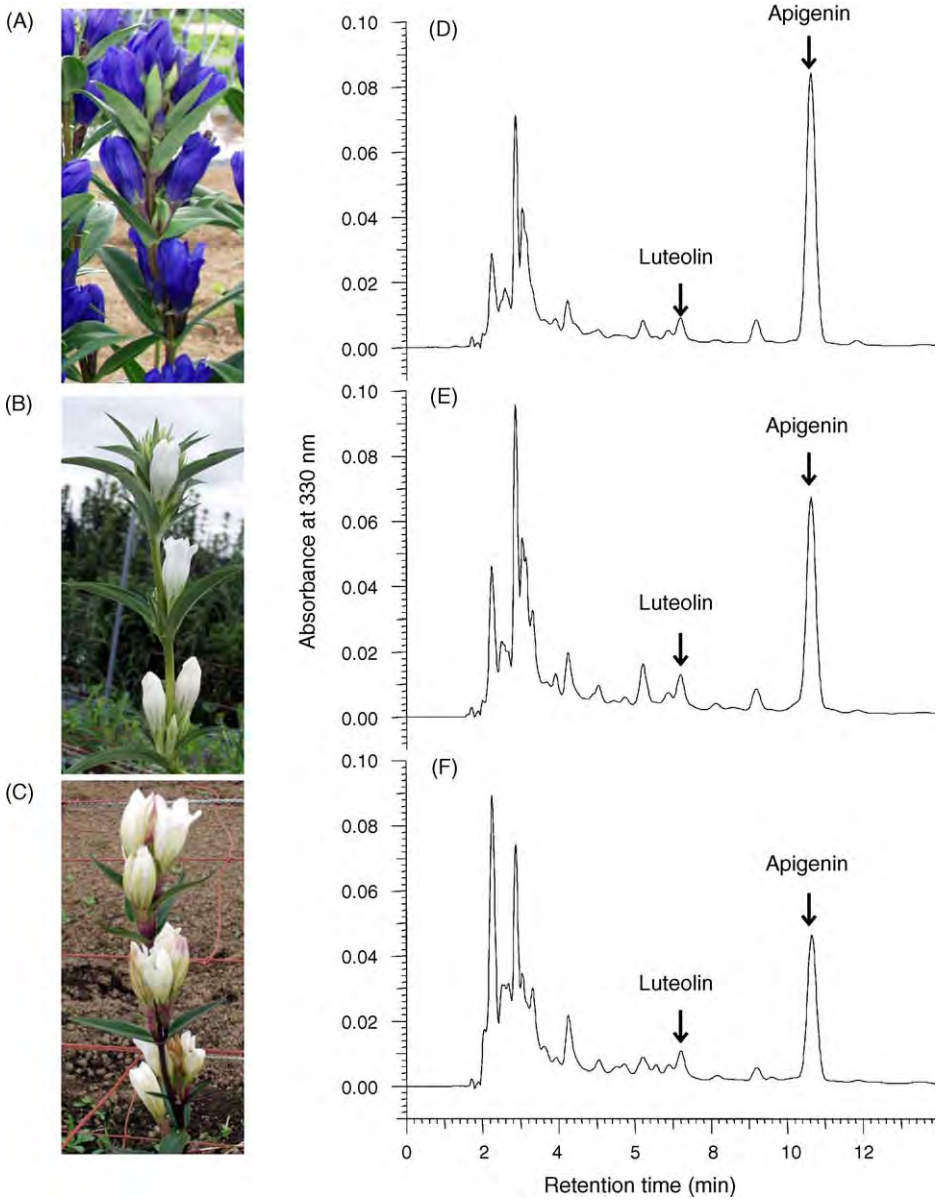

Fig. 1. Phenotypes and HPLC flavone profiles in blue- and white-flowered gentian cultivars. (A–C) The gentian cultivars used in this study: cv. Maciry, cv. Homoi and cv. Polano White, respectively. Flavone was extracted from the petals and subjected to HPLC analysis. (D–F) The flavone profiles of cv. Maciry, cv. Homoi and cv. Polano White, respecitvely.

similar to that of the blue-flowered cv. Maciry (Fig. 1). On the other hand, the quantity of flavone compounds in cv. Polano White petals (64.6 μg/g fresh weight) decreased by about half that in petals of cv. Maciry (100.0 μg/g fresh weight) and cv. Homoi (101.5 μg/g fresh weight). No flavonol derivatives were detected in the petals of all three cultivars (data not shown).

3.2. Expression analysis of flavonoid biosynthetic structural genes

The expression levels of 10 flavonoid biosynthesis structural genes (*CHS, CHI, F3H, F3′H, F3′,5′H, DFR, ANS, 3GT, 5AT* and *FSII*) were compared among petals of the blue- and white-flowered cultivars at two flower developmental stages (S1 and S3, Fig. 2). Northern blot analysis indicated that all tested genes were expressed in cv. Maciry petals at the unpigmented bud stage (S1) and/or just before anthesis (S3). The expression profiles of these genes in the petals of cv. Homoi were similar to those in cv. Maciry except for the *ANS* gene. In addition, reverse transcription-polymerase chain reaction (RT-PCR) analysis using several primer pairs did not amplify *ANS* transcripts in cv. Homoi (data not shown). These results suggest that the white coloration of cv. Homoi arose from suppression or deficiency of the *ANS* gene. On the other hand, no or weak *CHS, F3H, F3′,5′H, DFR, ANS, 3GT* and *5AT* transcripts were detected in the petals of cv. Polano White compared with cv. Maciry.

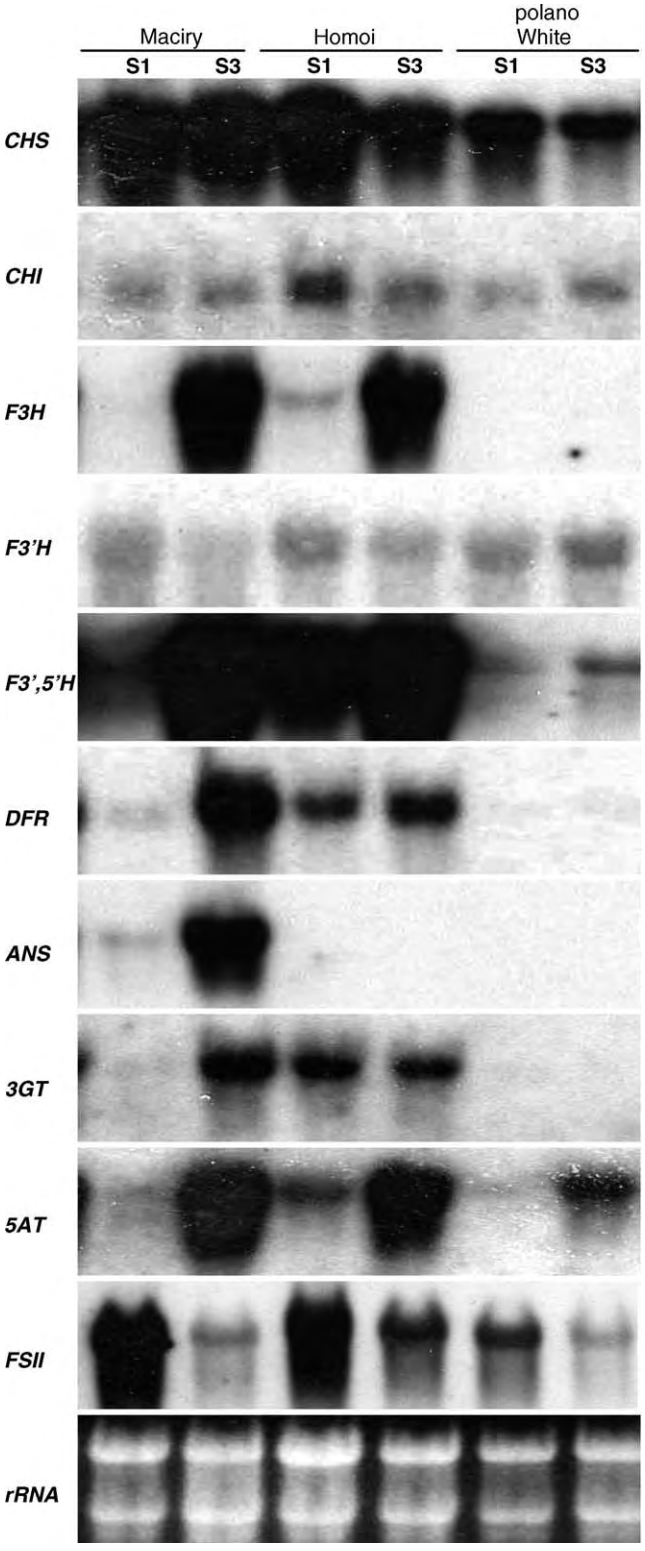

Fig. 2. Expression analyses of flavonoid biosynthesis structural genes in the petals of blue- and white-flowered gentian cultivars. Northern blot analysis was performed using total RNA (5 μg) from gentian petals at flower developmental stages 1 (S1) and 3 (S3), as defined in the Section 2. The blot was hybridized by probes for *CHS, CHI, F3H, F3′H, F3′,5′H, DFR, ANS, 3GT, 5AT* and *FSII* genes, respectively. Ribosomal RNA bands stained with ethidium bromide are shown as the control.

3.3. Genomic structure of the ANS gene in cv. Homoi and its parents

Because we were unable to amplify the *ANS* genomic sequence of cv. Homoi using several primer pairs designed from the *ANS* cDNA sequence of cv. Maciry, only the genomic *ANS* sequence of cv. Maciry was determined after PCR amplification of the *ANS* ORF region (accession no. AB208689). The result showed that cv. Maciry genomic *ANS* contains two exons and one 840 bp intron located 516 bp from the translation initiation site. To gain an insight into the mutation found in cv. Homoi, the structure of the *ANS* gene in the gentian genome was further analyzed by Southern blot analysis using cv. Maciry *ANS* cDNA as a probe (Fig. 3). Since cv. Homoi was bred as an F_1 hybrid from white-flowered parents, the parents were also subjected to analysis to reveal the origin of the mutation. The *ANS* probe labeling the ORF of *ANS* cDNA from cv. Maciry was hybridized using genomic DNA from each cultivar digested by either *Hin*dIII or *Eco*RI restriction enzymes. Blue-flowered cv. Maciry showed a single *Hin*dIII fragment (approximately 3.5 kbp) and four *Eco*RI fragments (approximately 6.5, 8, 9 and 11 kbp, respectively), indicating the presence of two loci in the genome (Fig. 3). On the other hand, two of the *Eco*RI fragments (approximately 8 and 9 kbp) were absent in the white-flowered cv. Homoi, and the *Hin*dIII fragment showed weaker signal intensity

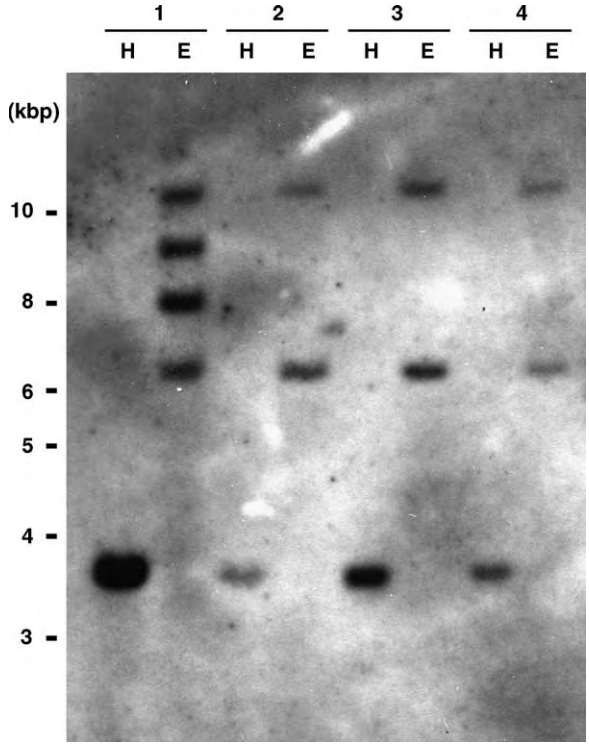

Fig. 3. Southern blot analysis of anthocyanidin synthase (*ANS*) gene. Total genomic DNA (10 μg) was digested by either *Hin*dIII (H) or *Eco*RI (E) then hybridized by a probe with the open reading frame (ORF) of *ANS*. (1) cv. Maciry; (2) cv. Homoi; (3) the maternal line of cv. Homoi; and (4) the paternal line of cv. Homoi. DNA size markers are indicated on the left.

compared to in cv. Maciry. The parents of cv. Homoi also showed the same pattern as shown by Southern blot analysis. Since the *ANS* genomic sequence contains an intron at *Eco*RI, but not *Hin*dIII, these results suggest that cv. Homoi completely lost one of the two *ANS* loci existing in the blue-flowered cv. Maciry, and that this deficiency was inherited as a null mutant of the same *ANS* locus derived from both parents.

3.4. Recovery of anthocyanin accumulation in the petals of cv. Homoi by particle bombardment

To determine whether the white coloration of cv. Homoi flowers arose from the absence of *ANS* transcripts, we performed a complementation test by transient *ANS* expression using particle bombardment. p35S-GtANS, in which cv. Maciry *ANS* cDNA was driven by the CaMV35S promoter, and p35S-sGFP as an internal transformation control, were co-precipitated onto gold particles and bombarded into abaxial petals of cv. Homoi just before anthesis. After 72-h incubation, red spots were easily observed against the white background in some epidermal cells under binocular microscope observation (Fig. 4A). Close-up magnification confirmed that the individual red spots were derived from single cells (Fig. 4B), which showed GFP fluorescence under UV microscopy (Fig. 4C), indicating that anthocyanin formation in cv. Homoi petals could be complemented by transient expression of *ANS* cDNA. When bombarded with p35S-sGFP alone, no red color spots were observed (data not shown).

3.5. Stress-induced anthocyanin accumulation in petals of cv. Polano White

Petals of cv. Polano White are usually ivory white, but they often turn red when exposed to environmental stresses such as cold and during senescence (Fig. 5A). Cold stress is known to induce pigmentation, and most gentian cultivars suffer induced pigmentation in their petals and leaves in late autumn, except the white-flowered cv. Homoi. HPLC analysis of cv. Polano White showed that this pigmentation was accompanied by anthocyanin production in the form of accumulating cyanidin derivatives in the petals (Fig. 5B) and leaves (data not shown). Total RNAs extracted from pigmented and unpigmented petal tissues of cv. Polano White at anthesis were subjected to northern blot analysis for flavonoid biosynthesis structural genes (Fig. 5C). *CHS, F3H, F3′H* and *ANS* transcripts were notably enhanced after induced pigmentation in the petals, whereas expression of *CHI, F3′,5′H* and *5AT* genes decreased. Similar results were

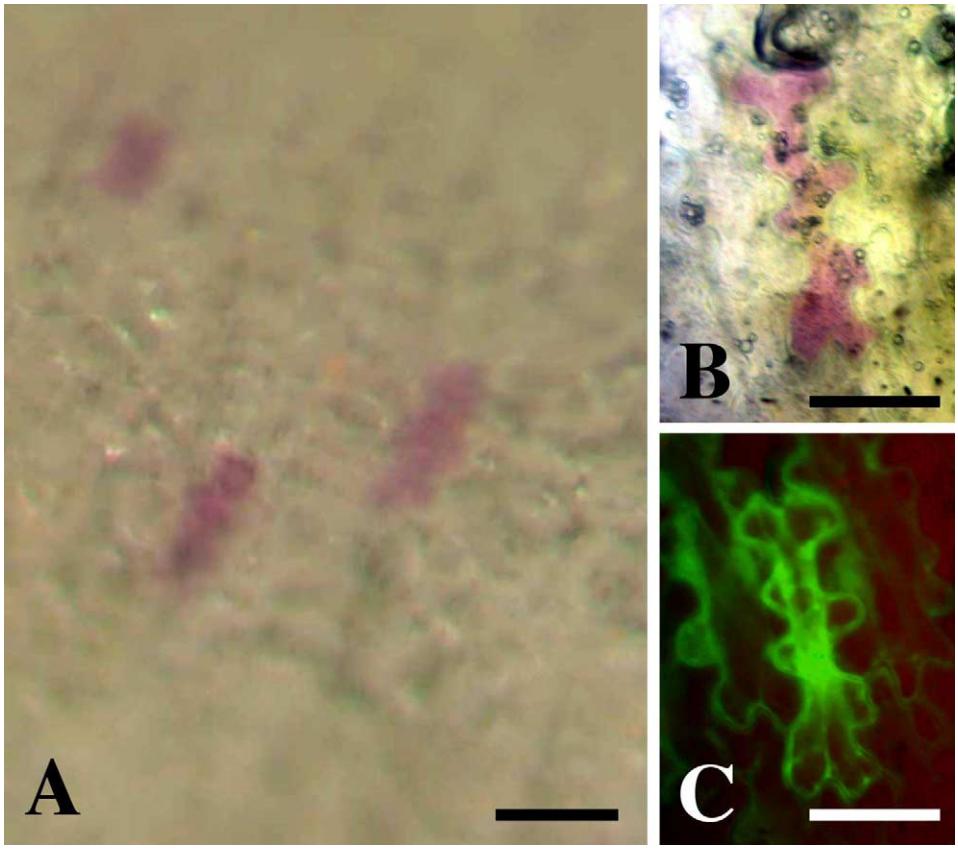

Fig. 4. Complementation test of the *ANS* gene in petals of cv. Homoi by particle bombardment. Petals of cv. Homoi were bombarded with gold particles coated with plasmid DNAs (p35S-GtANS and p35S-sGFP). After 3 days of bombardment, the petals were observed under a microscope (A); the scale bar indicates 100 μm. The pigmented cells were consequently observed under visible (B) or UV-B light (C); scale bars indicate 50 μm.

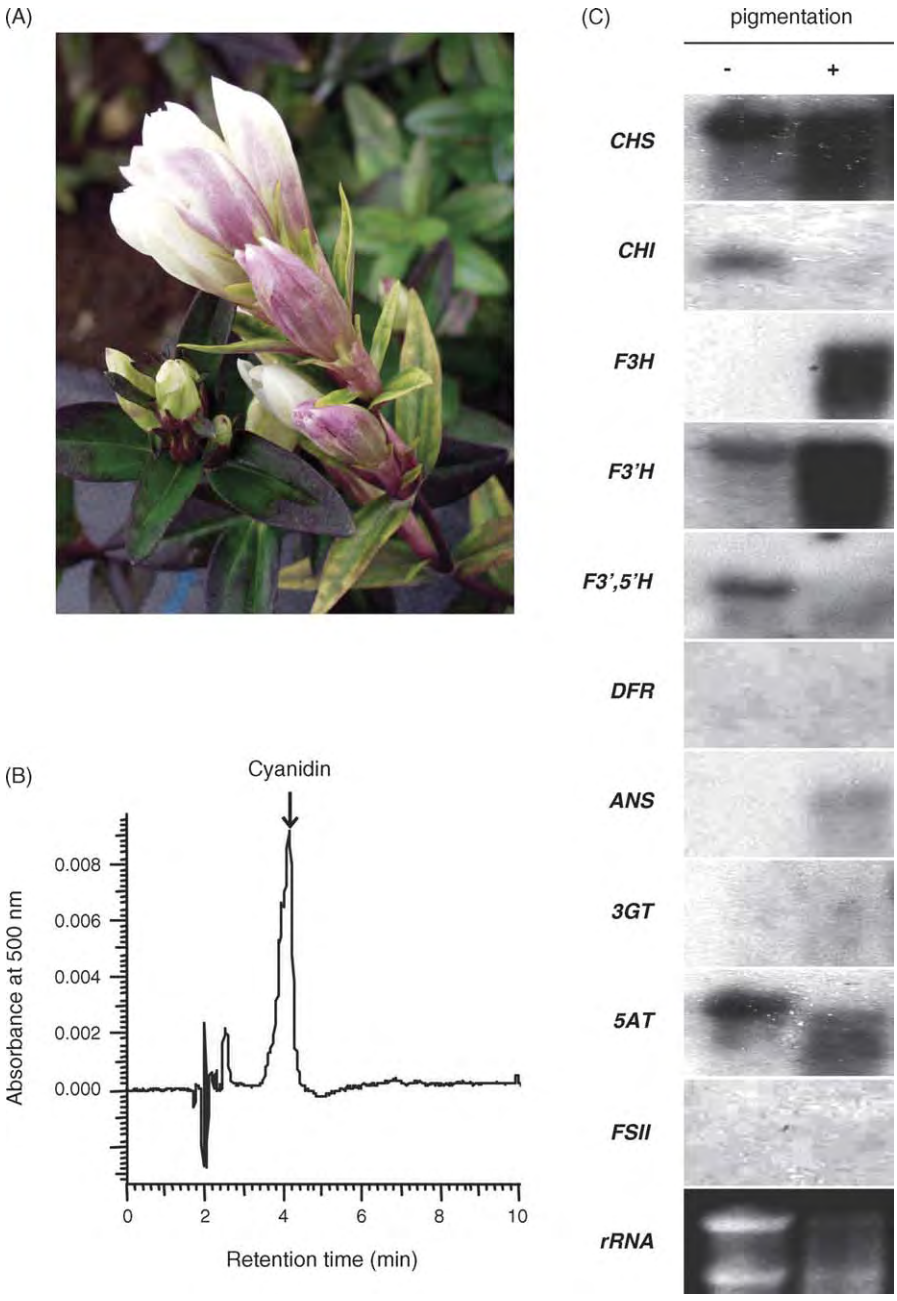

Fig. 5. Differential expression of flavonoid biosynthetic structural genes before and after pigmentation in the petals of cv. Polano White. (A) Pigment-induced flower of cv. Polano White. (B) Anthocyanidin extracts from pigmented petals were subjected to HPLC analysis and monitored at 500 nm, the maximum absorbance of anthocyanidin. (C) Northern blot analysis was performed using total RNA isolated from unpigmented (−) or pigmented petals (+).

observed by RT-PCR analysis using total RNA isolated from pigmented and unpigmented leaves of cv. Polano White (data not shown).

4. Discussion

In general, the flowers of native Japanese gentians are blue to purple in color as a result of an accumulation of delphinidin derivatives in the form of gentiodelphin [29]. Since most gentian cultivars have been bred using natural mutational resources, only a few flower color variations have been obtained, namely, blue, white, pink and pale blue, but not red or yellow colors. White-flowered cultivars have also been cross-pollinated with limited parent materials selected by gentian breeders, and therefore, most can be classified into one of two types based on their phenotype; one shows no pigmentation throughout (Homoi-type; Fig. 1B), and the other, Polano White-type, usually accumulates anthocyanin pigments in its stems, while its petals and leaves also show induced pigmentation as a result of environmental stress (Figs. 1C and 5A).

We previously demonstrated that flavonoid biosynthesis structural genes are temporally regulated in the petals of the blue-flowered gentian cv. Maciry allowing accumulation of anthocyanin and flavone compounds, and the coloration of blue flowers was characterized by biochemical and molecular biological analysis [1]. In this study, we attempted to characterize the molecular biological difference between white- and blue-flowered gentian cultivars. Because white-flowered cultivars are considered of commercial value, it is important to understand their hereditary background not only from the point of view of conventional breeding but also that of molecular breeding. Pigment analysis showed that the white-flowered cv. Homoi lacks anthocyanin accumulations in its petals, but the amount of flavone derivatives was not reduced (Fig. 1E). Consequently, we hypothesized that cv. Homoi has a mutation of the later steps of flavonoid biosynthesis that include F3H. In cv. Polano White, on the other hand, not only was anthocyanin accumulation absent, but flavone accumulation in the petals was reduced (Fig. 1F), suggesting that this white cultivar has a different mutation from cv. Homoi.

Northern blot analysis of cv. Homoi showed no *ANS* gene transcripts, corresponding well with the unchanging flavone accumulation profiles (Figs. 1 and 2). Because this cultivar is an F_1 diploid hybrid, Southern blot analysis suggested that cv. Homoi lacks one of two *ANS* gene loci compared with blue-flowered cultivars (Fig. 3). This is also supported by the parental Southern blot analysis. However, the other *ANS* locus remains in its genome. It is speculated that this might be a minor or pseudo gene not expressed in all tested tissues. Transient expression of gentian *ANS* cDNA using particle bombardment showed complementation of anthocyanin accumulation (Fig. 4), indicating direct evidence that anthocyanin accumulation in cv. Homoi petals disappeared due to an ANS activity deficiency. Furthermore, suppression of the *ANS* gene in transgenic blue-flowered gentians was previously shown to induce white flower phenotypes (Nishihara et al., unpublished results), indicating the definite role of the *ANS* gene in blue coloration. Although leucoanthocyanidin is thought to accumulate in the petals of cv. Homoi as a result of an *ANS* null mutation, this accumulation was not detected after acidification of flavonoid extracts with HCl (data not shown). Therefore, in cv. Homoi petals, leucoanthocyanidin might be converted to compounds other than anthocyanidin, such as condensed tannins catalyzed by anthocyanidin reductase [37]. *ANS* locus mutations have also been identified as *candica* in *A. majus* [5] and *E. grandiforium* [14], which possess ivory flowers, and *P. nil*, which has pale white flowers [10].

Northern blot analysis showed no or weak transcripts of not only the *ANS* gene but also *CHS, F3H, F3′,5′H, DFR, 3GT* and *5AT* in the petals of cv. Polano White (Fig. 2). However, Southern blot analysis showed the presence of all tested structural genes (data not shown), suggesting that the white coloration of cv. Polano White was not simply caused by mutation of structural genes. This is also supported by the fact that cv. Polano White has a pigmented stem (Fig. 1C) and suffers induced pigmentation (Fig. 5A). The above genes suppressed in cv. Polano White petals correspond with those expressed at later stages of flower development in blue-flowered gentians [1]; therefore, it is likely that cv. Polano White lacks activity of common regulatory factors controlling expression of genes induced during later steps of flavonoid biosynthesis. Similarly, some white-flowered lines of *Eustoma*, which, like gentians, belongs to the *Gentianaceae* family, show inactivity of regulatory factors controlling expression of genes induced during later steps of flavonoid biosynthesis [14].

Although the flowers of cv. Polano White are usually white, they often turn red under environmental stress. In autumn, senescing leaves of many temperate deciduous plants turn brilliant red [38,39], as do the flowers and leaves of most gentian cultivars. Induced pigmentation in cv. Polano White was accomplished by a change in gene expression related to flavonoid biosynthesis (Fig. 5). Expression of *CHS, F3H, F3′H* and *ANS* was recovered, while that of *CHI, F3′,5′H* and *5AT* was reduced. Since the anthocyanin derivative induced by environmental stress was shown in the form of cyanindin not delphinidin derivatives (Fig. 5B), suppression of *F3′,5′H* and *5AT* seems reasonable. Fujiwara et al. [27] reported that the main anthocyanin in the stems and sepals of gentians is in the form of cyanidin 3-glucoside.

The relationship between light and anthocyanin biosynthesis has been extensively studied [5,40], but that between low temperature and anthocyanin biosynthesis has only been studied using seedlings of maize [41] and *Arabidopsis* [42]. Maize seedlings incubated at a low temperature showed increased anthocyanin pigmentation and transcripts of some structural genes of flavonoid biosynthesis. These findings demonstrated that anthocyanin structural gene products accumulate during cold temperatures and gain optimal enzymatic activity only when seedlings are shifted to higher temperatures [41]. Although, in this study, we were unable to measure the pigmentation of white-flowered gentians with the time course of cold treatment, in future molecular analysis we hope to reveal the induction mechanism of anthocyanin.

In conclusion, our results demonstrate that two different spontaneous mutations are involved in white flower formation in cultivated gentian plants. These findings provide a substantial foundation for production of gentian plants with new flower colors by both conventional and molecular breeding, although further work is needed for a complete understanding of the mutations causing white coloration in gentians, especially Polano White-type flowers.

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