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Original Paper

Dominant inheritance of white-flowered and herbicide-resistant traits in transgenic gentian plants

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Abstract To produce white-flowered gentian plants, we attempted to suppress the chalcone synthase (*chs*) gene by *Agrobacterium*-mediated transformation. A binary vector, pSMABcCHS, harboring antisense cDNA of *chs* isolated from the gentian *Gentiana triflora* cv. Maciry under control of the CaMV35S promoter was transformed into an interspecific hybrid gentian (cv. Albireo; *G. scabra*×*G. triflora*). The vector also contained the *bar* gene as a selectable marker. Three out of seventeen transgenic plants showed completely white flowers with 10 to 25% reduced anthocyanin content compared with the wild-type. Molecular analyses confirmed integration of the foreign genes and suppression of *chs* mRNA accumulation in their petals. Application of commercial herbicide including bialaphos showed that the transformants were strongly resistant. Segregation of the white-flowered and herbicide-resistant traits was tested using a T_1 progeny obtained from crossing with a blue-flowered parental line. The results clearly showed that these two traits are inherited dominantly as linked traits in the T_1 progeny, suggesting that these transgenic plants are useful resources for production of white-flowered gentians. These results also demonstrated for the first time the inheritance of foreign genes and genetic modification of flower color in transgenic gentian plants.

Key words: Anthocyanin, chalcone synthase, gentian, white flowers.

Gentians are one of the most popular ornamental flowers in Japan with more than half of all gentian production being carried out in Iwate prefecture. *Gentiana triflora*, *G. scabra* and their hybrids are commonly cultivated and used as cut flowers and potted plants (Kohlein 1991). They usually exhibit blue flowers containing anthocyanin from delphinidin derivatives, namely gentiodelphin (Goto et al. 1982) and albireodelphin (Hosokawa et al. 1997a). Recently, white-flowered cultivars have also been produced through conventional breeding using natural mutations. These white-flowered gentian plants are valuable with market demands increasing not only in Japan but also overseas. However, since there are few genetic resources of white-flowered traits (Nakatsuka et al. 2005a), it is difficult to produce elite white-flowered gentians by conventional breeding. In addition, because white flower phenotypes are usually inherited as recessive traits, this increases the difficulty in breeding white flowered gentians. Thus, in response to the growing market, genetic engineering technology could be very useful in increasing white-flowered variation. Furthermore, genetic engineering is also very attractive for production of gentian plants with new flower colors such as red and yellow, further opening up the market of gentians.

Gentian flower color is the result of flavonoids, the biosynthesis-related genes of which have been cloned and analyzed extensively (Tanaka et al. 1996; Kobayashi et al. 1998; Fujiwara et al. 1998; Fukuchi-Mizutani et al. 2003; Nakatsuka et al. 2005b, 2006). Of these genes, chalcone synthase (CHS) has been shown to be an early key enzyme in flavonoid biosynthesis derived from the phenylpropanoid pathway. This enzyme catalyzes the condensation of three malonyl-CoA and one *p*-coumaroyl-CoA into naringenin chalcone (Heller and Forkmann 1993), and their regulation mechanisms have been studied intensively (Koes et al. 1990; Fritze et al. 1991; Kaiser et al. 1995; Bieza and Lois 2001; Wade et al. 2001). Accordingly, we cloned gentian *chs* cDNA and its promoter and revealed their flower-specific expression (Kobayashi et al. 1998). *chs* genes have also been used for modification of flower color in several higher plants.

Abbreviations: CHS, chalcone synthase; MS medium, Murashige and Skoog medium; PCR, polymerase chain reaction.

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For example, production of white or light colored flowers by suppression of *chs* using a sense or antisense strategy has been successfully achieved in petunia and tobacco (van der Krol et al. 1988; Napoli et al. 1990), chrysanthemum (Couryney-Gutterson et al. 1994), torenia (Aida et al. 2000; Suzuki et al. 2000) and gerbera (Elomma et al. 1993). The production of transgenic white-flowered lisianthus in the *Gentianaceae* family has also been reported (Deroles et al. 1995).

We therefore attempted to suppress *chs* in blue-flowered gentians in order to produce a white-flowered variety. In this study, we report successful production and characterization of white-flowered gentian plants by genetic transformation with antisense *chs*. We also analyze the T_1 progeny for inheritance of the introduced traits. This is the first report to demonstrate not only a change in flower color but also genetic inheritance of foreign genes in transgenic gentian plants.

Materials and methods

Plant materials

Plants of the interspecific hybrid gentian cultivar Albireo (*Gentiana triflora*×*G. scabra*) were maintained *in vitro* as described by Hosokawa et al. (2000). Shoot cultures were used for the transformation study. The blue-flowered parental line *G. triflora* was used for crossing with transgenic gentian plants.

Construction of a binary vector for suppression of gentian CHS

Full-length cDNA encoding gentian *chs* derived from *G. triflora* cv. Maciry (Kobayashi et al. 1998) was inserted into the binary vector pSMAB704 (Igasaki et al. 2002) in antisense orientation between the CaMV35S promoter and nopaline synthase terminator. The resulting plasmid, pSMABcCHS, was transferred into *Agrobacterium tumefaciens* strain EHA101 by electroporation for use in the present study.

Production of transgenic gentians

Gentian transformation was performed as described by Mishiba et al. (2005). Briefly, leaf segments (approx. 3×6 mm) were excised from *in vitro* cultured plants and subjected to *Agrobacterium* inoculation. After 1 week cocultivation, they were transferred onto selection medium containing 2 μg ml^{-1} bialaphos (Meiji-Seika co. Ltd., Tokyo, Japan) and 500 μg ml^{-1} cefotaxime (Claforan; Hoechst AG). Subcultures were conducted every two weeks by transferring the leaf explants onto fresh selection medium. Adventitious shoots were induced from the bialaphos-resistant calli then transferred to rooting medium containing 500 μg ml^{-1} cefotaxime. Acclimatization of the regenerated plants was performed according to the procedures reported by Hosokawa et al. (2000).

Confirmation of transgenic plants

Southern blot analysis was performed as described by Nakatsuka et al. (2005b) using total genomic DNA extracted from putative transformants. DIG-labeled PCR probes were used for hybridization. The primers used were as follows: for *chs*, 5′-TATGGCACCTTCCCTT-GATG-3′ and 5′-AAGAAGGGTTTGGGCTGCTG-3′; for *bar*, 5′-GGATCCATGAGCCCAGAACG-3′ and 5′-GCGGTTCCACCTCACCATTG-3′.

Anthocyanin accumulation in petals of transgenic gentian

To measure anthocyanin amounts in the petals of transgenic gentian lines, anthocyanin compounds were extracted with methanol containing 0.1% hydrochloric acid. Anthocyanin concentrations were estimated by measuring the absorbance of extracts at 530 nm using a spectrophotometer. Three to five flowers per each line were used for these measurements.

Accumulation of chs transcripts in transgenic gentian plants

Northern blot analysis was performed using stage four petals as defined by Nakatsuka et al. (2005b). DIG-labeled *chs* PCR probes were also used and hybridization, membrane washing and detection procedures were performed as described by Nakatsuka et al. (2005b).

Segregation of herbicide-resistance and white petal coloration in the T_1 progeny

Transgenic gentian line no. 12, which showed a clear reduction of blue pigmentation in its petals, was used for crossing with the blue parental line with reciprocal combinations. In the first experiment, the obtained T_1 seeds were aseptically sown on a germination medium consisting of MS basal medium supplemented with 3% sucrose and 0.2% gellan gum, and grown for about 2 months. Two leaf segments from each seedling were transferred onto selection medium containing 2 μg ml^{-1} bialaphos and cultured for 1 month under dim light conditions at 20°C. The seedlings were also subjected to PCR for *bar* amplification using the following primers: 5′-CGGCGGTCTGCACCATCGTCAACCACTACATC-3′ and 5′- GATGACAGCGACCACGCTCTTGAAGCC-CTGTG-3′. The seedlings were then acclimatized and grown in a closed greenhouse until flowering.

In the second experiment, the obtained T_1 seeds were sown directly onto soil, grown for about 2 months then sprayed with 200-fold diluted commercial herbicide (‘Herbi-ekizai’; Meiji-Seika Co. Ltd.) and cultivated in a closed greenhouse until flowering.

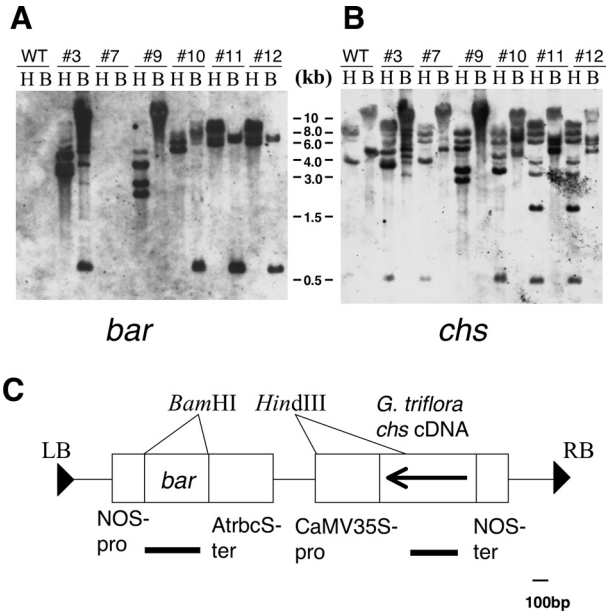

Figure 1. Southern blot analyses of putative transgenic gentian plants. A) Total genomic DNA was digested with *Hin*dIII (H) or *Bam*HI (B) then hybridized with *bar* using a probe. B) The membrane shown in panel A was reprobed with *chs*. DNA size markers are indicated in the center. C) The vector map used for transformation. RB: right border, LB: left boarder, CaMV: Cauliflower mosaic virus, NOS; nopaline synthase, *bar*; phosphinothricin acetyl transferase (resistance to bialaphos), AtrbcS; *Arabidopsis* Rubisco small subunit, pro; promoter, ter; terminator. The thick bars under the map show the probes for *chs* and *bar*, respectively.

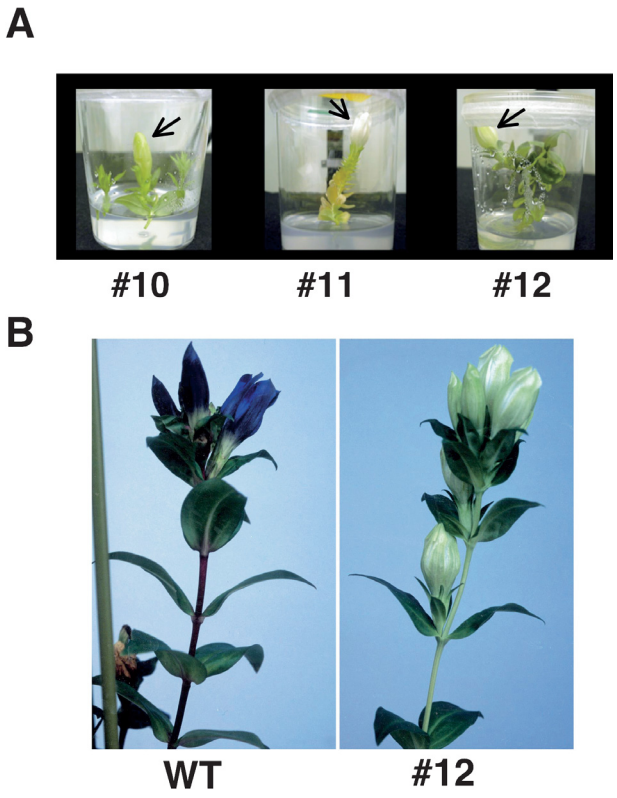

Figure 2. Phenotype of *chs*-suppressed transgenic gentian plants. A) Flowering *in vitro*. Arrows indicate flowers. B) Flowering in a closed greenhouse. WT: untransformed control plant, #10 to 12: transgenic lines.

Results

Production of transgenic gentian plants

A total of 21 bialaphos-resistant shoots were obtained after several transformation experiments using more than 10,000 leaf segments. Integration of foreign genes was checked by Southern blot analysis. Typical results of Southern blot analysis are shown in Figure 1. When probed with the *bar* gene, all putative transgenic plants except line no. 7 showed signals corresponding to the foreign *bar* gene. When the blot was reprobed with *chs*, several bands were newly detected in addition to the endogenous *chs* bands (shown in the WT lane) in all lines except line no. 7. Finally, 17 transgenic gentian plants were obtained and grown *in vitro* until flowering.

Phenotype of the transgenic gentian plants

Three out of the 17 transgenic gentian plants showed white flowers *in vitro* as shown in Figure 2A. After acclimatization, they grew normally and set white flowers in a closed greenhouse. Figure 2B shows a typical transgenic plant at flowering. The transgenic petals turned completely white compared with the vivid blue of the wild-type. Reduced pigmentation was also observed in the stems. After overwintering at 4°C, newly generated shoots from the transgenic plants were subjected to herbicide treatment. Application with commercial herbicide containing bialaphos showed that the transformants were strongly resistant unlike untransformed control plants, which died within one month (data not shown).

Accumulation of anthocyanin and chs transcripts in petals of transgenic plants

Three transgenic gentian plant lines (nos. 10, 11 and 12) with completely white flowers were further analyzed for accumulation of anthocyanin and *chs* mRNA in their petals (Figure 3). Pigment analysis showed that the anthocyanin contents of all three lines were reduced by up to 90% reduction compared to the wild type (Figure 3A). Northern blot analysis also showed that accumulation of *chs* transcripts was completely suppressed in the petals of all three transgenic lines (Figure 3B).

Inheritance of white flowers and herbicide-resistant traits in the progeny

Viable seeds were obtained from crossing with a blue-flowered parental line then grown under two conditions. In the first experiment, herbicide resistance was tested by observing the regeneration ability of leaf segments excised from *in vitro* grown seedlings on medium

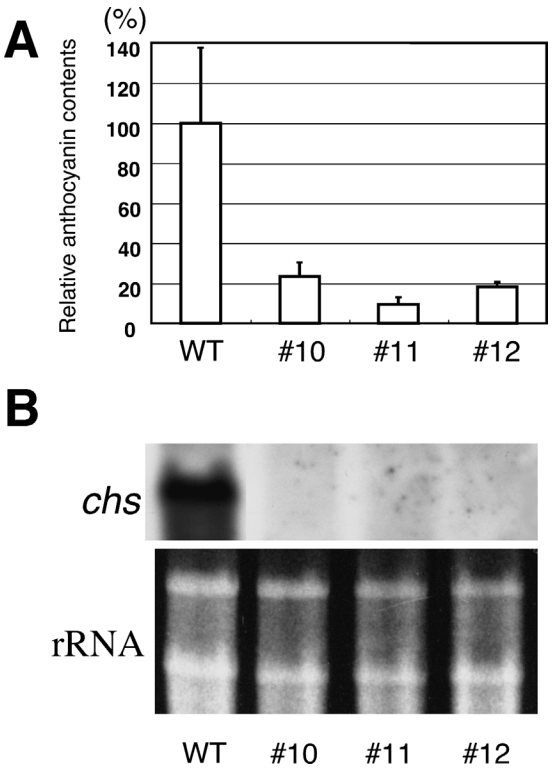

Figure 3. Accumulation of anthocyanin and *chs* mRNA in transgenic gentian plants. A) Anthocyanin contents in the petals of transgenic (lines #10 to #12) and untransformed control plants (WT) as determined by spectrophotometry. Values indicate averages of three to five flowers±standard deviations. B) Northern blot analysis of the petals of transgenic (lines #10 to #12) and untransformed control plants (WT) probed with *chs*.

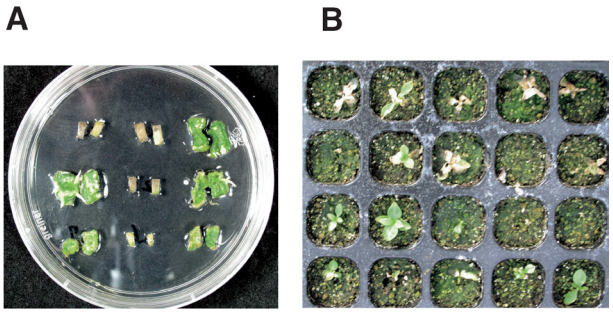

Figure 4. Bialaphos-resistance test of the T_1 progeny. A) Leaf segments one month after culture initiation; segments excised from T_1 seedlings were transferred on regeneration medium containing 2 mg l^{-1} bialaphos. B) Thirty-day old seedlings one month after treatment with herbicide ('Herbi-ekizai') containing bialaphos.

containing bialaphos. The seedlings were also subjected to PCR analysis for detection of the foreign *bar* gene. About one-half of the seedlings showed bialaphos-resistant traits (Figure 4A). This resistance was coincident with the presence of the *bar* gene confirmed by PCR (Table 1). The seedlings were then cultivated in pots and their flower colors were observed. As shown in Figure 5A, they set either white or blue flowers; that is, bialaphos resistant-seedlings all set white flowers and

Table 1. Inheritance of the *bar* gene and bialaphos resistance.

	bar	
	+	−
PCR analysis	33	46
Bialaphos-resistance	33	0

Bialaphos-resistance was checked by regeneration on bialaphos-containing medium as described in the Materials and methods.

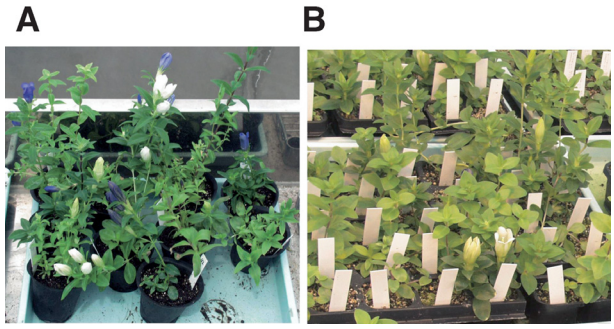

Figure 5. Flowering of the T_1 progeny. A) T_1 seedlings without herbicide selection set blue or white flowers. B) T_1 seedlings with herbicide selection set white flowers only.

sensitive-seedlings all set blue flowers.

In the second experiment, the seeds were directly sown in a well tray and treated with herbicide containing bialaphos as shown in Figure 4B. The surviving seedlings were grown until flowering; all set white flowers as shown in Figure 5B. These results indicate dominant inheritance of white flower color and herbicide-resistance in the T_1 progeny.

Discussion

White flowered-gentian plants were successfully produced in this study. Suppression of the *chs* gene using an antisense method has been reported previously in petunia and tobacco (van der Krol et al. 1988), gerbera (Elomma et al. 1993), lisianthus (Deroles et al. 1995) and torenia (Aida et al. 2000). Thus, we applied this strategy to gentians, one of the most important ornamental flowers in Japan. Though gentians are a resistant species for genetic transformation, we established a practical genetic transformation system using *Agrobacterium* (Hosokawa et al. 1997b; Mishiba et al. 2005) and particle bombardment (Hosokawa et al. 2000) through a trial and error process. Molecular analyses clearly confirmed the presence of the introduced foreign genes (*bar* and *chs*) and suppression of *chs* mRNA in the white-flowered transgenic gentian lines. Analyses of protein levels such as the amount and activity of chalcone synthase will be needed for further characterization of these transformants.

In most cases, white flowered phenotypes are known to be recessive traits because they are caused by blocking

of the flavonoid biosynthetic pathway. For example, white flowers have been reported resulting from a *niv* mutation in snapdragon (Spribille and Forkmann 1992) and *f* mutation in stock (Rall and Hemeleben 1984), both of which affect chalcone synthase. Ivory white flowers have also been reported to have mutations in flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR) or anthocyanidin synthase (ANS) in snapdragon (Martin and Gerats 1991; Martin et al. 1985; Martin et al. 1991), morning glory (Saito et al. 1994; Hoshino et al. 1997), carnation (Stich et al. 1992; Mato et al. 2000) and lisianthus (Davies et al. 1993). In the case of Japanese cultivated gentian, white flowers are known to be derived from mutants involved in anthocyanidin synthase (ANS) or, perhaps, transcriptional factor(s) (Nakatsuka et al. 2005a). Our transgenic plant with suppressed *chs* represents another source of white flowers in gentians. *Chs* suppression is also thought to produce pure white flowers due to the absence of flavone, a major unpigmented flavonoid in gentians. In addition, *chs*-suppressed transgenic gentian plants have an advantage over previously developed cultivars due to the linked dominant inheritance of the white flower color and herbicide-resistant traits, as demonstrated in this study. Furthermore, since the commercial herbicide used in this study is usually used by gentian growers, this herbicide-resistant trait will be of high value in gentian cultivation.

Flavonoids are also known to be involved in many biological functions such as attraction of pollinators, UV protection and defense responses against environmental stress in higher plants (Mol et al. 1998; Winkel-Shirley 2001). Because *chs*-suppressed transgenic gentian plants do not accumulate flavone and anthocyanin, major flavonoids in blue-flowered gentians, sensitivity to environmental stress such as UV should be tested in field trials in the future. However, as far as we observed in the closed greenhouse experiment, there was no difference between transgenic and control plants with regard to growth and flowering. More noteworthy, our transgenic gentians set seeds after crossing of reciprocal pollination combinations with the wild-type. It has been reported that flavonol reduction in pollen due to lack of CHS protein causes conditional male fertility in maize and petunia (Mo et al. 1992; Pollak et al. 1993; Napoli et al. 1999). It is likely that the activity of the CaMV35S promoter is weak in pollen or anther tissues compared with petal tissues in gentian plants. On the other hand, our recent study showed that the CaMV35S promoter in transgenic gentian plants is strictly subjected to methylation-associated transgene silencing (Mishiba et al. 2005). To confirm whether the *chs* suppression was caused transcriptionally or post-transcriptionally, further study (e.g. analysis of epigenetic status in trans- and endo-*chs* gene regions) will be needed.

In conclusion, we demonstrated that genetic engineering is a promising approach for modification of flower color in gentians. However, the transformation frequency was not so efficient in this study; that is, only 3 out of 17 transgenic lines turned white. RNA interference (RNAi)-mediated silencing is becoming an increasingly efficient silencing technique in plants as well as other organism (Wesley et al. 2001; McGinnis et al. 2005), and down-regulation of chalcone synthase genes with RNAi has been successfully achieved in torenia (Fukusaki et al. 2004). Thus, we are currently attempting to produce *chs*-suppressed gentian plants by RNAi-mediated silencing. Comparative studies between antisense and RNAi are also now in progress.

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