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Cosuppression of tobacco chalcone synthase using *Petunia* chalcone synthase construct results in white flowers

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ABSTRACT. Chalcone synthase (CHS; EC 2.3.1.74) is a key enzyme in anthocyanin biosynthesis. In order to understand the molecular mechanism controlling flower color, tobacco plants were transformed with a chimeric construct containing expression cassettes for neomycin phosphotransferase II (*nptII*) selection marker and CaMV 35S promoter-driven *Petunia chsA* cDNA, via *Agrobacterium*-mediated method. Four transformants produced white flowers, while three transformants produced pink flowers similar to the untransformed parent. Thin layer chromatography analysis revealed the absence of cyanidin in all white-flowered transformants. Northern blot analysis showed that total *chs* mRNA levels were greatly decreased in white-flowered transformants. By contrast, *chs* mRNA expression was induced in pink-flowered transformants. RT-PCR analysis showed that the relative level of endogenous tobacco *chs* mRNA was less than that of the transgenic *Petunia chsA* mRNA in white-flowered lines. In addition, plant/T-DNA junction sequence analysis excluded the possibility that insertion of T-DNA into anthocyanin genes had inactivated the anthocyanin biosynthetic pathway in white-flowered tobacco plants. Taken together, these results indicate that cosuppression of the tobacco *chs* gene can occur using the equivalent *Petunia* gene, and demonstrate a linkage between the expression level of *chs* mRNA, cyanidin content, and flower color in transgenic tobacco plants.

Keywords: Anthocyanin; Chalcone synthase; Cosuppression; Flower color modification; *Nicotiana tabacum*; Transgenic plants; Transgene silencing.

Abbreviations: CaMV, cauliflower mosaic virus; **CHS,** chalcone synthase; **GUS,** β-glucuronidase; **NOS,** nopaline synthase; **NPTII,** neomycin phosphotransferase II; **RT-PCR,** reverse transcription-polymerase chain reaction; **T-DNA,** transfer DNA; **TLC,** thin layer chromatography.

INTRODUCTION

Flower color is largely determined by two classes of pigments: flavonoids, which contribute to a range of colors from yellow to red to blue to purple; and carotenoids, which are responsible for the red, orange and yellow lipid-soluble pigments found embedded in the membranes of chloroplasts and chromoplasts (Bartley and Scolnik, 1995). Anthocyanins are a major colored class of flavonoids that are responsible for the pink, red, violet and blue colors of flowers and other tissues. Anthocyanins perform diverse roles like attracting pollinators and dispersing fruits and seeds. They also play key roles in the signaling that takes place between plants and microbes, in the male fertility of some species, in defense as antimicrobial agents and feeding deterrents, and in UV protection (Dixon and Steele, 1999; Forkmann and Martens, 2001; Winkel-Shirley, 2001). Three common anthocyanins are the pelargonidin-based (brick red to orange), cyanidin-based (pink to red), and delphinidin-based (blue) pigments.

The anthocyanin biosynthetic pathways of higher plants—including those in *Petunia*, maize, snapdragon, and recently *Arabidopsis*—are all well established (Holton and Cornish, 1995; Mol et al., 1998; Winkel-Shirley, 2001). Briefly, chalcone synthase (CHS; EC 2.3.1.74) catalyzes condensation of one molecule of *p*-coumaroyl-coenzyme A (CoA) and 3 molecules of malonyl-CoA, resulting in one molecule of 4, 2', 4', 6'-tetrahydroxychalcone (chalcone), which is a key intermediate in the formation of flavonoids. The CHS

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enzyme, usually found in plant epidermal cells, has a molecular weight of about 42,000 Da, requires no cofactors, and has been isolated from several plant species, including French bean, parsley, and carnation (Seigler, 1998). More recently, three-dimensional structure and functional studies of alfalfa CHS enzyme have shown that four residues (Cys164, Phe215, His303, Asn336) in the catalytic site are responsible for its decarboxylation and condensation reactions, and are highly conserved among different species (Ferrer et al., 1999; Jez et al., 2000).

The first *chs* gene to be cloned was isolated from parsley by an immunological approach (Kreuzaler et al., 1983). The maize *c2* gene, encoding a CHS enzyme expressed in the aleurone layer of the kernel, was isolated using transposon tagging (Wienand et al., 1986). Additional *chs* genes have been isolated using hybridization to cDNA libraries or genomic libraries with the previously isolated *chs* clones. In *Petunia hybrida* inbred line V30, 8 to 10 copies of *chs* genes, with high homology (approximately 80%) at the DNA level, have been identified (Koes et al., 1989). RNase protection analysis with gene-specific probes have shown that only two members, *chsA* and *chsJ*, are expressed in anthers and corollas (Koes et al., 1989; Quattrocchio et al., 1993) and that *chsJ* transcripts are 5% to 20% that of *chsA* levels (Koes et al., 1989; Gutterson, 1995; Napoli et al., 1999). In soybean, a total of eight *chs* genes have recently been reported, but molecular characterization for each member has not yet been done (Tuteja et al., 2004). Expression of *chs* has been well studied in a number of other plant species. In early developmental stages of oat plants this enzyme is present in leaf tissue (Knogge et al., 1986), in contrast to adult *Petunia* plants, where its presence is limited to floral tissue (Koes et al., 1986; Koes et al., 1989). Environmental stress, such as UV light, phytopathogens and elicitors, or wounding, leads to an induction of *chs* gene expression (Koes et al., 1989; Winkel-Shirley, 2002). Developmental, tissue-specific and inducible regulation of *chs* genes makes it an interesting system with which to study the molecular mechanisms underlying plant gene regulation.

Flower color is one of the most important characteristics in ornamental plant breeding. By controlling expression levels of genes involved in or regulating the anthocyanin biosynthesis pathway, novel varieties with respect to flower color have been obtained from several plants (Forkmann and Martens, 2001; Schijlen et al., 2004). The gene encoding the CHS enzyme is one target that can be manipulated for just such a purpose. Here we report the characterization of flower color through introduction of a *Petunia chsA* cDNA into tobacco, a model species for plant transformation. We found that four out of seven transgenic plants possessed flowers that were altered from pink to white. Genetic analysis and molecular characterization of these transformants were carried out, and they suggested a role for cosuppression in these white flowers.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of tobacco (*Nicotiana tabacum* cv. W38) and *Petunia hybrida* cv. Ultra Blue were sown in a mixture of peat and vermiculite and grown in a growth chamber under a cycle of 14-h (6:00 a.m. to 8:00 p.m., 25°C) illumination (250 μmol/m^2/s) and 10-h (8:00 p.m. to 6:00 a.m., 20°C) darkness. After 1 month, plantlets were moved into a green house and grown until maturation.

Plasmid construction and plant transformation

The transforming vector pCHS carrying expression cassettes for neomycin phosphotransferase II (*nptII*) selection marker and cauliflower mosaic virus (CaMV) 35S promoter-driven *Petunia chsA* was constructed as described (Wang and To, 2004). This vector was used to transform tobacco via *Agrobacterium tumefaciens* strain LBA4404 as described (Horsch et al., 1985). After selection on medium containing 100 μg/ml kanamycin sulfate, regenerated plants were transferred to pots and grown in a green house. Integration of the construct into the tobacco genome was confirmed by PCR analysis as described (Wang and To, 2004).

Genomic blot analysis

Genomic DNA from green leaves of tobacco was isolated as previously described (To et al., 1999). DNA restriction enzyme digests, Southern blotting, the synthesis of probes containing CaMV 35S promoter and full-length *Petunia chsA* cDNA, hybridization and detection were described by Wang and To (2004).

Northern blot analysis

Total RNA was extracted from different tissues with acid guanidinium thiocyanate-phenol-chloroform, Northern analysis was performed and probed with full-length *Petunia chsA* cDNA as previously described (To et al., 1999; Huang et al., 2001; Wang and To, 2004). The mRNA levels were quantified using X-ray films analyzed with a chemiluminescent analyzer (IR LAS-100 Lite, Fujifilm, Japan).

Extraction and TLC analysis of anthocyanins

Four flowers from each transgenic line and wild-type tobacco were pooled and used for anthocyanin extraction as described (Oud et al., 1995) with modification. Petals were crushed with a mortar and pestle in 2 ml of 1% (v/v) HCl in methanol. The methanol-HCl solution was lyophilized. Anthocyanin pigments were dissolved in 0.8 ml of 2 N HCl and incubated for 30 min at 100°C. The solution was cooled on ice and centrifuged for 2 min at 13,000 rpm. The supernatant was transferred into a new Eppendorf tube. One hundred μl of isoamylalcohol was added, and the mixture was vortexed vigorously for 2 min. The organic phase was separated and stored at 4°C until use.

For TLC analysis, 2 µl of isoamylalcohol extract was spotted onto a cellulose plate (TLC Plastic Sheets Cellulose, Merck) using a micro capillary. The mobile phase was acetic acid-water-HCl (30:10:3) (v/v). Pigment standards of pelargonidin, cyanidin, and delphinidin were purchased from Apin Chemicals (Oxfordshire, UK).

Seedling assay for kanamycin resistance

T1 seeds from self-fertilized transgenic plants were sterilized in 1% sodium hypochloride for 20 min, and washed thoroughly with sterile distilled water. They were then germinated on a selection medium containing MS salts, 3% sucrose, 0.8% agar, and 100 µg/ml kanamycin sulfate. The cultures were incubated at 25°C under 14-h illumination for 3 to 4 weeks. Seedlings with white cotyledons, no true leaf development, and inhibition of root extension were considered to be kanamycin sensitive while seedlings with green cotyledons and healthy development of leaves and roots were considered to be kanamycin resistant.

RT-PCR analysis

Total RNA from different tissues was treated with DNase I and 100 ng of treated total RNA was used to perform reverse transcription-polymerase chain reaction (RT-PCR) analysis, using a one-step RT-PCR kit (Qiagen). For amplification of species-specific *chsA* regions, primers P1 (5′-CAGTGAGCACAAGACTGAT-3′) and P2 (5′-GAGATGGCCATCAATAGCA-3′) specific for *Petunia* (accession no. AF233638), and primers T1 (5′-ACGGTACTCCGGATGGCT-3′) and T2 (5′-GAAATTCCCAAAGGTTGG-3′) specific for tobacco (accession no. AF311783), were employed simultaneously in the same PCR tube (Figure 5C). Reverse transcription was carried out at 50°C for 30 min. Initial PCR activation was carried out at 95°C for 15 min. DNA amplification was carried out for 30 cycles (denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min). Final extension was performed at 72°C for 10 min. For amplification of consensus the *chsA* region for both *Petunia* and tobacco plants, consensus primers C1 (5′-ACAACAAGGGCGCTCGAG-3′) and C2 (5′-CAAGCCCTTCACCAGTAG-3′) were employed (Figure 5F). Protocols for RT-PCR analysis were the same as mentioned above, with the exception that the annealing temperature for PCR amplification was changed from 55 °C to 57°C. Following amplification, 5 µl of PCR product was analyzed on a 1% agarose gel. Quantification of PCR products from RT-PCR analysis was conducted by analyzing photographs with a chemiluminescent analyzer (IR LAS-100 Lite, Fujifilm, Japan).

Cloning of plant/T-DNA insertion sequence

The strategy used for cloning plant/T-DNA insertion sequences by an inverse PCR-based method has been described elsewhere (Chen et al., 2003).

RESULTS

Transgenic plant verification

Seven transgenic plants were obtained. Genomic PCR analysis was carried out to confirm integration of the chimeric cassette into the tobacco chromosomal genome from *in vitro* plantlets using sequence-specific primers for *nptII* and *Petunia chsA* sequences (Figure 1A). No PCR products were detected in wild-type tobacco (W38) using *nptII*-specific or *Petunia chs* primers for amplification. A unique band of approximately 0.8 kb, which is the nearly expected size (795 bp) of the kanamycin resistant gene (*nptII*), was observed in all transgenic plants using the *nptII*-specific primers for amplification. A unique band of approximately 1.2 kb, the expected size (1,170 bp) of *Petunia chsA* cDNA, was also observed in all transgenic plants using the *Petunia chsA* primers for amplification, except for transformant CHS21 (right panel in Figure 1A).

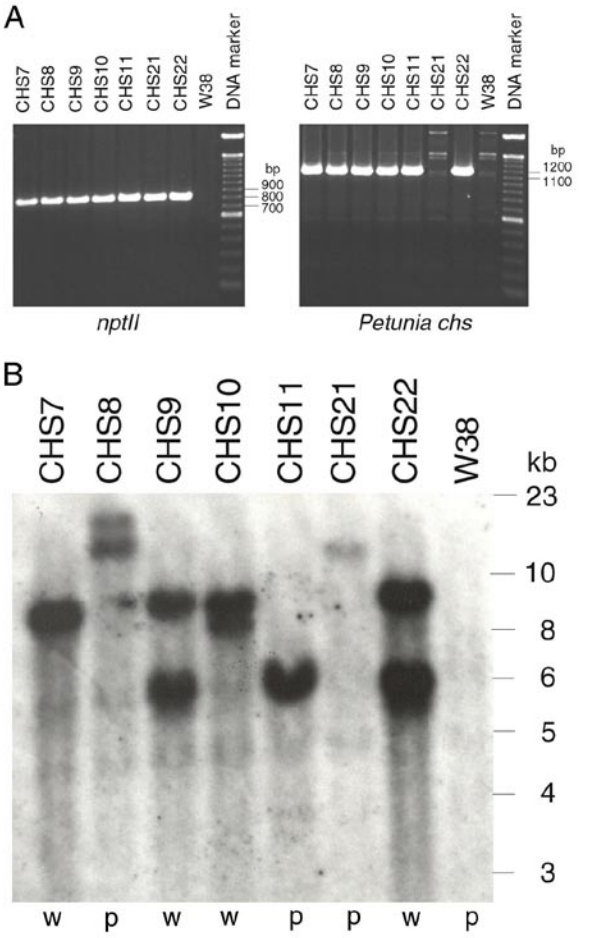

Figure 1. Confirmation of transgene in transgenic tobacco plants. (A) Genomic PCR analysis from wild-type tobacco (W38) as well as transgenic plants. (B) Southern blot analysis with non-radioactive DIG probe containing a CaMV 35S promoter and *Petunia chsA* cDNA. Labels "p" and "w" in this panel, as well as in Figure 3, Figure 4 and Figure 5, represent the flower phenotype "pink" and "white".

In vitro plantlets were transferred and grown in a greenhouse. Large-scale isolation of genomic DNA from green leaves and Southern blot analysis were used to estimate the transgene copy number of each plant (Figure 1B). No *Eco*RI site is present in *Petunia chsA* or tobacco *chsA* sequences. No hybridization band was detected in wild-type DNA digested with *Eco*RI and probed with the 2-kb DNA fragment containing CaMV 35S promoter and *Petunia chsA* cDNA, probably due to the presence of non-homologous CaMV 35S promoter (835 bp) in our probe and to washing conditions. After hybridization, the membrane was washed twice in washing solution (0.2% SSPE; 0.1% SDS) at 65°C for 10 min and once in buffer 1 (0.1 M maleic acid, pH 7.5; 0.15 M NaCl; 0.3% Tween 20) at room temperature for 20 min, as described previously (Wang and To, 2004). One hybridization band was detected in CHS7, CHS11 and CHS21 transformants, suggesting that only 1 copy of transgenic cassette was integrated into their genomes. Two hybridization bands were detected in CHS8, CHS9, CHS10 and CHS22 transformants, suggesting that there were two insertions in these transgenic lines.

Alteration of flower color in transgenic plants

The seven transgenic tobacco plants were grown to maturity. White flowers were observed in four transformants (CHS7, CHS9, CHS10 and CHS22), and pink flowers were observed in the remaining three (CHS8, CHS11 and CHS21) as well as in the wild-type tobacco control plant (W38) (Figure 2). No apparent phenotypes other than flower color were affected by the transformation.

Figure 2. Alteration of flower color in transgenic tobacco plants by overexpression of sense *Petunia* chalcone synthase construct. Seven transgenic plants were obtained, and four transformants (CHS7, CHS9, CHS10, CHS22) carried white flowers. Similar pink floral pigmentation as wild-type tobacco (W38) was observed in transformants CHS8, CHS11 and CHS21.

To further investigate the relationship between the accumulation of anthocyanidins and phenotypic variation in flower color of transgenic plants, thin layer chromatography (TLC) analysis was conducted (Figure 3). The major component of anthocyanins extracted from petals of wild-type pink flowers as well as from petals of three transformants carrying pink flowers (CHS8, CHS11, CHS21) was cyanidin. In contrast, transformants with white flowers (CHS7, CHS9, CHS10, CHS22) accumulated a very low to undetectable level of cyanidin, suggesting blockage of the cyanidin biosynthetic pathway.

Total *chs* mRNA is largely suppressed in white-flowered transgenic plants

Overexpression of chimeric sense or antisense *chs* constructs reducing floral anthocyanin accumulation has been reported elsewhere (Napoli et al., 1990; Courtney-Gutterson et al., 1994; Jorgensen et al., 1996; Metzlaff et al., 2000; Suzuki et al., 2000). To examine *chs* mRNA in transgenic plants, total RNA was isolated from petals and probed with the *Petunia chsA* sequence (Figure 4). A strong signal was detected in wild-type tobacco (W38) with the relative *chs* mRNA level in this sample set as indicating 100% *chs* mRNA expression. A similar *chs* mRNA expression level was detected in a white-flowered tranformant CHS9 (105%). *chs* mRNA was reduced in other white-flowered transformants (CHS7, 38%; CHS10, 50%; CHS22, 70%), but *chs* mRNA level was induced in pink-flowered transformants (CHS8, 351%; CHS11, 461%; CHS21, 143%).

Relative levels of transgenic and endogenous *chs* mRNA in transgenic plants

To further characterize and distinguish endogenous *chs* expression from foreign *Petunia chsA* mRNA in transgenic tobacco plants, nucleotide sequences of the *chs* coding region between *Petunia* and tobacco were compared (Figure 5A), and high similarities (91% identity) were observed. Two primer sets, specific for tobacco (T1 and T2 primers) or *Petunia* (P1 and P2 primers), were designed accordingly (Figure 5B). Total

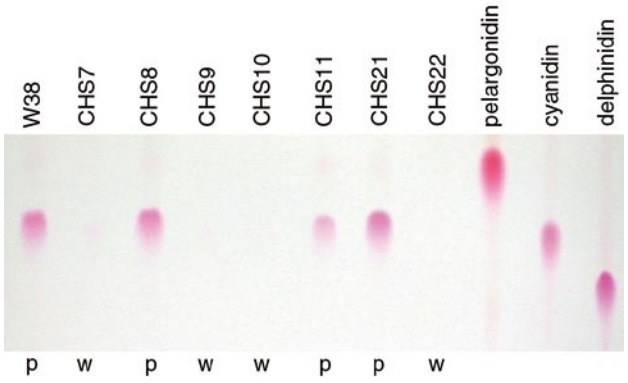

Figure 3. Thin layer chromatography analysis showing the absence of cyanidin in white-flowering transgenic plants.

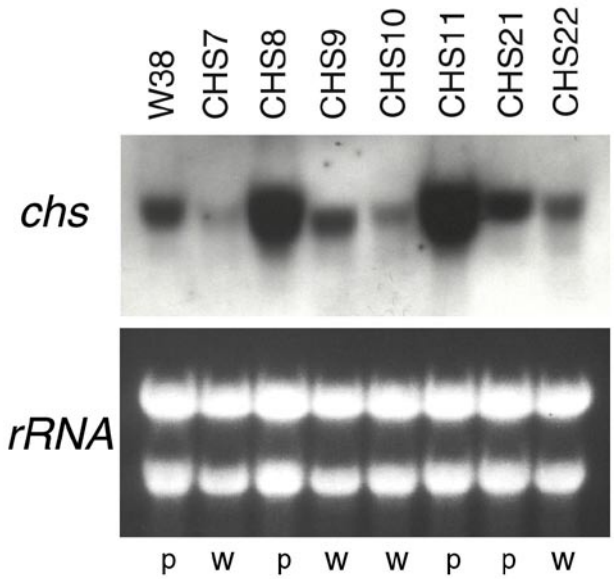

Figure 4. Northern blot analysis of the total *chs* mRNA level in transgenic plants.

RNA was isolated from petals in different transformants as well as wild-type plants, and species-specific primer sets were employed simultaneously to examine the relative levels of transgenic and endogenous *chs* mRNA by RT-PCR analysis (Figure 5C). Only tobacco-specific but not *Petunia*-specific *chs* mRNA was detected in wild-type tobacco plants, and only *Petunia*-specific but not tobacco-specific *chs* mRNA was detected in wild-type *Petunia* plants (Figure 5C). Two different transcripts representing the transgenic and endogenous *chs* mRNA were detected in all transgenic plants, with the exception that only endogenous tobacco-specific *chs* mRNA was detected in a pink-flowered transformant CHS21 (Figure 5C). Comparison of the relative percentages of transgenic and endogenous *chs* mRNA within each transgenic plant clearly showed that the intensity of a PCR band (approximately 0.4 kb) corresponding to tobacco-specific *chs* mRNA was obviously weaker in all white-flowered transformants (CHS7, 19%; CHS9, 15%; CHS10, 20%; CHS22, 15%) than in those pink-flowered transformants (CHS8, 37%; CHS11, 41%; CHS21, 100%). Higher levels of transgenic *Petunia*-specific *chs* mRNA, as revealed by *Petunia*-specific PCR product (approximately 0.65 kb), were detected in the pink-flowered transformants (CHS8, CHS11) (Figure 5C).

We also tested whether this gene construct was expressed in other tissues of transgenic plants. In brief, total RNA isolated from leaf tissue of transgenic plants and *Petunia*-specific P1/P2 primers were used in RT-PCR analysis (Figure 5D). A unique PCR product with the expected size (approximately 0.65 kb) was detected in six of the seven transformants. No PCR amplification was observed in transformant CHS21 or wild-type tobacco (W38) (Figure 5D).

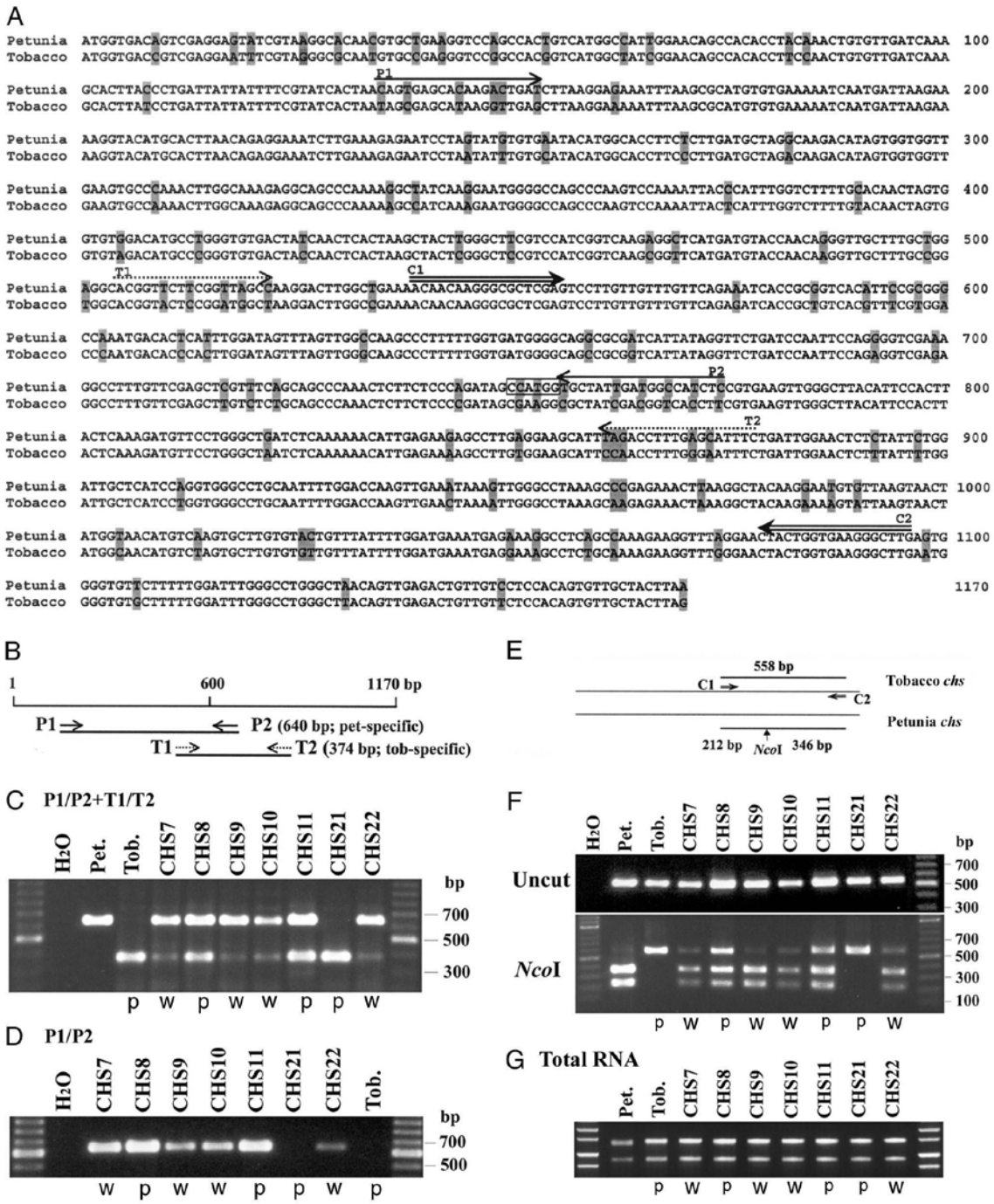

Figure 5. Sequence comparison of tobacco and *Petunia chs* cDNA and RT-PCR analysis showing cosuppression of endogenous *chs* mRNA in white-flowered transformants. (A) Nucleotide sequences of tobacco (accession no. AF311783) and *Petunia* (accession no. AF233638) cDNAs encoding chalcone synthase. Different nucleotides between *chs* sequence of these two plant species are highlighted in gray, and locations of primers are indicated by arrows. The recognition sequence for *Nco*I is boxed. (B) Strategy for identification of foreign *Petunia chs* mRNA expression in transgenic tobacco plants by RT-PCR analysis. (C) Relative levels of transgenic (640 bp) and endogenous (374 bp) *chs* mRNA by RT-PCR analysis. Endogenous *chs* mRNA was largely reduced in those white-flowered transformants (CHS7, CHS9, CHS10, CHS22). Abbreviations "Tob" and "Pet" in this panel, as well as in Figure 5D, Figure 5F, and Figure 5G represent "wild-type tobacco W38" and "wild-type *Petunia*". (D) Detection of foreign *Petunia chs* mRNA in the leaf tissue of transformants by RT-PCR analysis. (E) Strategy for confirmation of foreign *Petunia chs* mRNA expression in transgenic tobacco plants by RT-PCR analysis combined with restriction enzyme digestion. (F) Total RNA from petals of each sample was isolated, and the primer set C1/C2 was used to amplify *chs* transcripts from both tobacco and *Petunia chs* cDNA by RT-PCR analysis. *Nco*I was added to digest the *Petunia chs* PCR fragments. (G) 100 ng of DNase-treated RNA from each sample prior to RT-PCR was run as a loading control on a 1% RNA agarose gel.

To exclude the possibility that the differential intensity of RT-PCR products (Figure 5C) resulted from different Tm values for each primer set (Tm for P1 and P2 is 56°C and 58°C, respectively whereas Tm for T1 and T2 is 58°C and 52°C, respectively), another primer set C1/C2 (Tm for C1 and C2 is 58°C and 56°C, respectively) was designed to amplify the 558 bp fragment from both *chs* cDNA of tobacco and *Petunia* (Figure 5E). To differentiate between the two *chs* cDNAs, we used the fact that the *Petunia*-specific *chs* fragment contained a unique *Nco*I restriction site not found in the tobacco fragment. Digestion of the *Petunia* PCR fragment resulted in two smaller segments of 212 bp and 366 bp while the tobacco-specific *chs* fragment remained uncut at 558 bp due to the absence of a *Nco*I restriction site. Petal RNA samples were prepared from different transformants as well as the wild-type tobacco and *Petunia* plants. The consensus C1/C2 primer set was employed to examine the relative levels of transgenic and endogenous *chs* mRNA by RT-PCR analysis (Figure 5F). A unique band of approximately 558 bp was detected in all uncut samples examined. After purification, the PCR products were subjected to *Nco*I digestion. Two smaller fragments were observed in an RNA sample isolated from control *Petunia* plants; by contrast and as expected, only one band of approximately 558 bp was detected in the RNA sample isolated from the wild-type tobacco plants. We further examined the percentage of endogenous and transgenic *chs* gene expression in individual transgenic plants based on *Nco*I digestion (Figure 5F). In those white-flowered transformants, the relative mRNA level of endogenous *chs* gene (CHS7, 17%; CHS9, 7%; CHS10, 17%; CHS22, 22%) was found much lower than the transgenic *Petunia*-specific *chs* gene (CHS7, 83%; CHS9, 93%; CHS10, 83%; CHS22, 78%). By contrast, the relative mRNA level of the endogenous *chs* gene (CHS8, 37%; CHS11, 30%) was found to be around 50% the level of the transgenic *Petunia*-specific *chs* gene (CHS8, 63%; CHS11, 70%) in each pink-flowered transformant.

Inheritance in T1 progeny of transgenic plants

The stable integration of the chimeric construct into the tobacco chromosomal genome was investigated by germinating T1 seeds from self-fertilized transgenic plants on MS medium containing 100 μg/ml kanamycin sulfate (Table 1). Kanamycin-resistant and kanamycin-sensitive T1 seedlings segregated in a 3:1 (resistant:sensitive) ratio in three transformants (CHS7, CHS10, CHS22) and a 15:1 ratio in the other two transformants (CHS8, CHS11), suggesting the presence of single copy of *nptII* transgene in the nuclear genomes of three transformants (CHS7, CHS10, CHS22) and two copies of *nptII* transgene in the nuclear genomes of two transformants (CHS8, CHS11). More than two copies of the *nptII* transgene were found in transformant CHS21. No *nptII* transgene was found in transformant CHS9 or wild-type tobacco W38. In summary, transformant CHS9 was considered to be kanamycin sensitive, and the other six transformants were considered to be kanamycin resistant (Table 1). Since the transforming vector pCHS contains expression cassettes for the selection marker gene (*nptII*) and *Petunia chs* cDNA within the T-DNA region (Wang and To, 2004), it is reasonable to predict the existence of the same copy number of the *nptII* transgene and *Petunia chs* transgene in each transformant (Table 1).

Molecular cloning of plant flanking sequence

The *Agrobacterium*-mediated transformation has been useful for introducing new genes into plants and for inactivation of plant genes by T-DNA insertion mutagenesis (Tinland, 1996). We now employed an inverse PCR-based method (Chen et al., 2003) to determine the T-DNA insertion sequences in all white-flowered transformants (CHS7, CHS9, CHS10, CHS22) and one pink-flowered transformant CHS21, which did not express *Petunia chs* mRNA (Figure 5). A unique plant DNA sequence, near the right border of T-DNA,

Table 1. Alteration of flower color and inheritance in T_1 progeny assay of transgenic tobacco plants.[a]

Transformant line	Flower's color	No. of resistant and sensitive seedlings from T_0 selfed seeds		Test ratio (R:S)	χ^2	P	Predicted copy no. of *nptII* transgene
		R	S				
CHS7	White	125	43	3:1	0.0317	0.95~0.80	1
CHS8	Pink	277	18	15:1	0.0093	0.95~0.80	2
CHS9	White	0	198				0
CHS10	White	408	138	3:1	0.0165	0.95~0.80	1
CHS11	Pink	513	36	15:1	0.0899	0.8~0.7	2
CHS21	Pink	374	12				>2
CHS22	White	301	95	3:1	0.2155	0.7~0.5	1
Wild type W38	Pink	0	248				0

[a]Seeds germinated on medium containing 100 μg ml^{-1} kanamycin for 3 to 4 weeks.

was obtained for transformants CHS7, CHS9 and CHS10 (Figure 6). The flanking DNA sequence (266 bp) in transformant CHS7 had no sequence similarity to any public domain nucleotide or expressed sequence tag (EST) databases; however, the flanking sequence (287 bp) in transformant CHS10, which also had no sequence similarity to nucleotide databases, showed a high similarity to several EST sequences including DD6-4 (tobacco ESTs characterized by hypersensitive response specific pattern; E-value=1E-58), EST606585 (mixed potato tissue cDNA clone; E-value=2E-23), EST498621 (*P. infestans*-challenged leaf *Solanum tuberosum* cDNA clone; E-value=2E-23), EST580272 (potato roots *Solanum tuberosum* cDNA clone; E-value=1E-21) and EST895187 (*Lycopersicon esculentum* maturing fruit cDNA clone; E-value=4E-15). For transformant CHS9, a partial region (79 bp) of the plant insertion sequence (156 bp) was identical to the transgenic *Petunia chs* gene (complementary to nucleotide positions 1092 to 1170 in Figure 5A), indicating that the right border of one T-DNA construct carrying the chimeric expression cassette for *nptII* and *Petunia chsA* genes had been integrated into the 5′ end of the *Petunia chsA* gene in another T-DNA construct likely carrying the same chimeric expression cassette.

Three different inserts were detected in transformant CHS21. Surprisingly, the insert sequence (55 bp) in clone CHS21-1 was a partial sequence in the T-DNA left border region of the transforming vector pCHS while the insert sequence (321 bp) in clone CHS21-3 was also a partial vector sequence near the 5′ terminus of the T-DNA right border. The insert sequence (400 bp) in clone CHS21-2 showed no sequence similarity with transforming vector pCHS and was considered to be a plant DNA sequence. However, no sequence similarity was found in public databases. Two different inserts were detected in transformant CHS22. The insert sequence (194 bp) in clone CHS22-1 was a partial T-DNA sequence between the NOS terminator and the T-DNA left border. The insert sequence (55 bp) in clone CHS22-2 showed 100% identity to the tobacco intergenic spacer of the rRNA gene (GenBank accession no. D76443). No sequence similarity was found among the insertion sequences we obtained, supporting the hypothesis of random integration of T-DNA into plant chromosomal genomes (Tinland, 1996; Hellens et al., 2000).

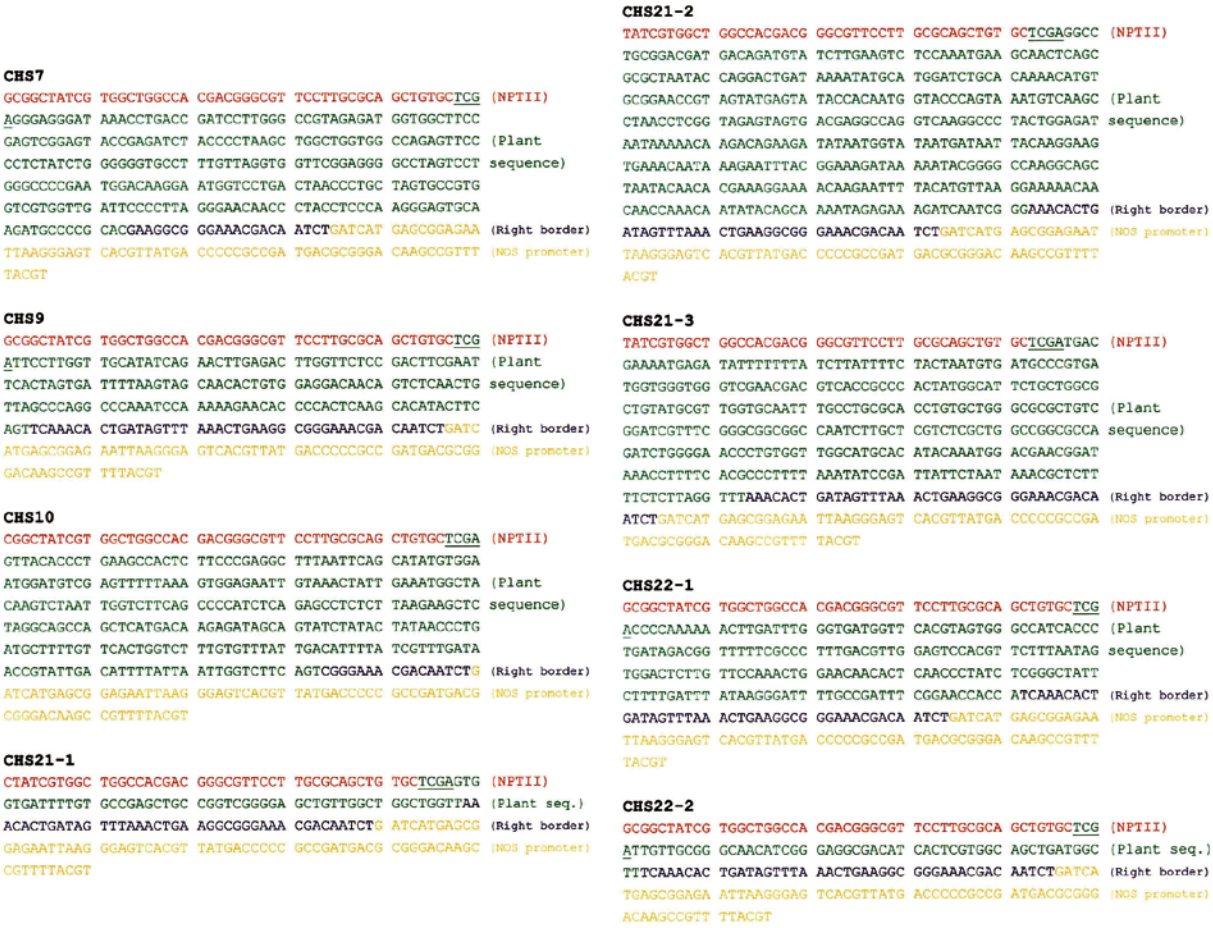

Figure 6. Inverse PCR analysis of selected transgenic tobacco plants showing plant/T-DNA insertion and relevant sequences. The *nptII* fragment (red), right border region (blue) and NOS promoter region (yellow), are derived from the transforming vector pCHS. The obtained tobacco sequence is indicated in green color, and the first *Taq*I restriction site within the tobacco sequence is underlined.

Taken together, partial sequences corresponding to T-DNA left border or right border were detected in transformants CHS9, CHS21 and CHS22, suggesting the presence of direct or invert repeats in their genomes. This is consistent with the observation that multiple T-DNA frequently integrate at the same position in plant genomes during *Agrobacterium*-mediated transformation, resulting in formation of inverted and direct repeats (De Buck et al., 1999). No sequence similarity was found between obtained plant sequences and any well-known pigment-related genes in databases. We thus conclude that loss of flower pigmentation in the white-flowered transformants we examined was not due to the inactivation of pigment-synthesizing genes in the host plant genome by T-DNA insertion.

DISCUSSION

In this study, a *Petunia chsA* transgene was introduced into tobacco, and its effect on flower colors in transgenic tobacco plants was investigated. Four out of seven transgenic plants showed phenotypic alteration from pink flowers to pure white flowers. It has been well documented that introduction of additional copies of *chs* gene into *Petunia* and *Arabidopsis* plants frequently results in events of *chs* post-transcriptional gene silencing (PTGS) or "cosuppression" (Depicker and Van Montagu, 1997; Metzlaff et al., 2000). Because CHS is the key enzyme in the anthocyanin biosynthetic pathway, silencing of this gene can be easily monitored by loss of pigmentation in flowers. Multiple models of PTGS have been proposed, including roles for RNA thresholds and DNA repeats (Baulcombe, 1996). In parallel, PTGS has been suggested to be due to involvement of a form of aberrant or double stranded RNA (Vaucheret et al., 1998; Chicas and Macino, 2001; Vaucheret et al., 2001).

Introduction of sense or antisense *chs* constructs has been carried out to modify flower color in various species (see below). However, to the best of our knowledge, introduction of sense chimeric *chs* into tobacco plants has not been reported. van der Krol et al. (1988) reported that when antisense *Petunia chs* cDNA was introduced into tobacco plants, 36 out of 40 had flowers indistinguishable from wild-type tobacco flowers; three plants gave flowers with sectored pigmentation; and one plant gave completely white flowers. The effect of overexpressing a sense *Petunia chs* cDNA in *Petunia* plants on flower color patterns has also been studied extensively. van der Krol et al. (1990) reported that six transgenic plants were obtained from *P. hybrida* var. VR (violet) using sense *Petunia chs* construct whilst no phenotypic changes were observed in any of the transgenic flowers; however, two of these plants, when grown under higher light conditions, produced flowers with patches of white floral tissue. In contrast, Napoli et al. (1990) reported that three out of six transformants from a *Petunia* commercial hybrid variety (Pink Cascade) produced pure white flowers. A progeny assay from one of these white-flowering transformants demonstrated that the novel color phenotype co-segregated with introduced *chs* whereas progeny plants without the transformed gene showed a wild-type phenotype. RNase protection analysis of petal RNAs isolated from white flowers showed that the level of the *chs* mRNA was reduced 50-fold from wild-type plants (Napoli et al., 1990). Molecular breeding to generate white flowers has been attempted in the high-value medicinal plant *Echinacea purpurea* (Wang and To, 2004) and in several ornamental plants, including *Dendranthema grandiflora* (Courtney-Gutterson et al., 1994), *Eustoma grandiflorum* (Deroles et al., 1998), *Gerbera hybrida* (Elomaa et al., 1993), *Torenia fournieri* (Aida et al., 2000), and *T. hybrida* (Suzuki et al., 2000) using sense or antisense *chs* constructs; however, only *D. grandiflora* (florist's chrysanthemum), *E. grandiflorum* (lisianthus flowers), and *T. hybrida* have produced white-flowered transformants. These experiments and our present study show that genetic modification of flower color can be accomplished by transforming chimeric or endogenous *chs* constructs into plants. However, effects on color modification are not easily predicted and can differ between plant species and varieties of the same species.

The kanamycin-sensitive phenotype in the white-flowered transformant CHS9 in this study is interesting. The kanamycin-resistant gene (*nptII*) is present in this transformant (Figure 1A). Moreover, integration of the chimeric cassette into tobacco genome was demonstrated by Southern blot analysis (Figure 1B) and the plant/T-DNA insertion site was characterized (Figure 6). *Petunia*-specific *chs* mRNA was also detected by RT-PCR analysis of this transformant (Figures 5C and 5F). An explanation for this observation may include preferential silencing of the foreign *nptII* gene but not the foreign *Petunia chs* gene within the chimeric construct of this transformant. Since *Petunia*-specific *chs* mRNA could be detected in both flower and leaf tissues from this transformant (Figure 5), it represents an excellent resource for studying the characteristics and mechanisms of transgene-induced gene silencing in plants (Gelvin, 1998; Vaucheret et al., 1998; De Wilde et al., 2000; Chicas and Macino, 2001; Vaucheret et al., 2001; Han and Grierson, 2002). Another transformant CHS21 is also interesting. This line shows normal levels of the endogenous *chs* transcription (Figure 5) and cyanidin content (Figure 3) and produces normal pink flowers. In addition, this line had detectable levels of the transgenic *nptII* gene (left panel in Figure 1A) and was resistant to kanamycin (Table 1) but showed no detectable levels of the transgenic *Petunia chs* gene (right panel in Figure 1A) and its corresponding transgenic *chs* transcript (Figure 5), suggesting that only a partial *Petunia chs* gene may have been transferred into the plant genome during the T-DNA integration process.

Northern blot analysis of total *chs* mRNA levels (Figure 4) together with RT-PCR analysis (Figure 5) demonstrated cosuppression of *chs* mRNA occurred in white-flowered transformants while overexpression of *chs* mRNA

occurred in pink-flowered transformants. Overexpression of *chs* sense or *chs* antisense suppression resulting in white-flowered transformants has been reported previously (Napoli et al., 1990; Courtney-Gutterson et al., 1994; Jorgensen et al., 1996; Metzlaff et al., 2000; Suzuki et al., 2000). TLC analysis of anthocyanidin accumulation (Figure 3) showed that loss of flower color in our transgenic plants (CHS7, CHS9, CHS10, CHS22) was linked to a dramatic reduction in *chs* mRNA levels (Figure 4). In contrast, the content of cyanidin in transformants with pink flowers (CHS8, CHS11, CHS21) with overexpressed *chs* mRNA levels, was found to be similar (CHS8, CHS21) or even lower (CHS11) than wild-type plants. Since the deduced amino acid sequence of the *Petunia* and tobacco CHS shows 95% identity, it is possible that *Petunia* CHS enzyme could be catalytically active in transgenic tobacco. Chalcone (the product of the CHS reaction) and naringenin (the product of the CHI reaction), are not only important for synthesizing different kinds of anthocyanins, but they are also key intermediates for synthesizing different flavonoids (Dixon and Steele, 1999; Forkmann and Martens, 2001; Winkel-Shirley, 2001). It has been reported that overexpression of *Petunia* chalcone isomerase (the enzyme catalyzes chalcone to naringenin) in tomato produced an increase of up to 78-fold in fruit peel flavonols, mainly due to an accumulation of rutin; however, no gross phenotypic differences were observed between high-flavonol transgenic and control lines (Muir et al., 2001). It is possible that the overexpression of the *Petunia chs* gene in transgenic tobacco plants may also lead to the synthesis and then accumulation of other types of flavonoids (most of which are colorless) while maintaining steady amounts of anthocyanins and, as a result, could generate flowers with a similar (pink) color. Further experiments of flavonoid profiling and the use of species-specific antibodies which can distinguish *Petunia* and tobacco CHS enzymes will be useful in understanding flower color modification by chimeric *chs* gene expression.

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LITERATURE CITED

Aida, R., S. Kishimoto, Y. Tanaka, and M. Shibata. 2000. Modification of flower color in torenia (*Torenia fournieri* Lind.) by genetic transformation. Plant Sci. **153:** 33-42.

Bartley, G.E. and P.A. Scolnik. 1995. Plant carotenoids: pigments for photoprotection, visual attraction, and human health. Plant Cell **7:** 1027-1038.

Baulcombe, D.C. 1996. RNA as a target and a initiator of post-transcriptional gene silencing in transgenic plants. Plant Mol. Biol. **32:** 79-88.

Chen, P.Y., C.K. Wang, S.C. Soong, and K.Y. To. 2003. Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. Mol. Breed. **11:** 287-293.

Chicas, A. and G. Macino. 2001. Characteristics of post-transcriptional gene silencing. EMBO Rep. **2:** 992-996.

Courtney-Gutterson, N., C. Napoli, C. Lemieux, E. Firoozabady, and K.E.P. Robinson. 1994. Modification of flower color in florist's chrysanthemum: production of a white-flowering variety through molecular genetics. Bio/Technology **12:** 268-271.

De Buck, S., A. Jacobs, M. Van Montagu, and A. Depicker. 1999. The DNA sequences of T-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. Plant J. **20:** 295-304.

De Wilde, C., H. Van Houdt, S. De Buck, G. Angenon, G. De Jaeger, and A. Depicker. 2000. Plants as bioreactors for protein production: avoiding the problem of transgene silencing. Plant Mol. Biol. **43:** 347-359.

Depicker, A. and M. Van Montagu. 1997. Post-transcriptional gene silencing in plants. Curr. Opin. Cell Biol. **9:** 373-382.

Deroles, S.C., J.M. Bradley, K.E. Schwinn, K.R. Markham, S. Bloor, D.G. Manson, and K.M. Davies. 1998. An antisense chalcone synthase gene leads to novel flower patterns in lisianthus (*Eustoma grandiflorum*). Mol. Breed. **4:** 59-66.

Dixon, R.A. and C.L. Steele. 1999. Flavonoids and isoflavonoids-a gold mine for metabolic engineering. Trends Plant Sci. **4:** 394-400.

Elomaa, P., J. Honkanen, R. Puska, P. Seppänen, Y. Helariutta, M. Mehto, M. Kotilainen, L. Nevalainen, and T.H. Teeri. 1993. *Agrobacterium*-mediated transfer of antisense chalcone synthase cDNA to *Gerbera hybrida* inhibits flower pigmentation. Bio/Technology **11:** 508-511.

Ferrer, J.L., J.M. Jez, M.E. Bowman, R.A. Dixon, and J.P. Noel. 1999. Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. Nat. Struct. Biol. **6:** 775-784.

Forkmann, G. and S. Martens. 2001. Metabolic engineering and applications of flavonoids. Curr. Opin. Biotechnol. **12:** 155-160.

Gelvin, S.B. 1998. The introduction and expression of transgenes in plants. Curr. Opin. Biotechnol. **9:** 227-232.

Gutterson, N. 1995. Anthocyanin biosynthetic genes and their application to flower color modification through sense suppression. HortScience **30:** 964-966.

Han, Y. and D. Grierson. 2002. Relationship between small antisense RNAs and aberrant RNAs associated with transgene mediated gene silencing in tomato. Plant J. **29:** 509-519.

Hellens, R., P. Mullineaux, and H. Klee. 2000. A guide to *Agrobacterium* binary Ti vectors. Trends Plant Sci. **5:** 446-451.

Holton, T.A. and E.C. Cornish. 1995. Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell **7:** 1071-1083.

Horsch, R.B., J.F. Fry, N.L. Hoffman, D. Eicholts, S.G. Rogers, and R.T. Fraley. 1985. A simple and general method for transferring genes into plants. Science **227:** 1229-1231.

Huang, Y.J., K.Y. To, M.N. Yap, W.J. Chiang, D.F. Suen, and S.C.G. Chen. 2001. Cloning and characterization of leaf senescence up-regulated genes in sweet potato. Physiol. Plant. **113:** 384-391.

Jez, J.M., J.L. Ferrer, M.E. Bowman, R.A. Dixon, and J.P. Noel. 2000. Dissection of malonyl-coenzyme A decarboxylation from polyketide formation in reaction mechanism of a plant polyketide synthase. Biochemistry **39:** 890-902.

Jorgensen, R.A., P.D. Cluster, J. English, Q. Que, and C.A. Napoli. 1996. Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA. Plant Mol. Biol. **31:** 957-973.

Knogge, W., E. Schmelzer, and G. Weissenbock. 1986. The role of chalcone synthase in the regulation of flavonoid biosynthesis in developing oat primary leaves. Arch. Biochem. Biophys. **250:** 364-372.

Koes, R.E., C.E. Spelt, H.J. Reif, P.J.M. van den Elzen, E. Veltkamp, and J.N. Mol. 1986. Floral tissue of *Petunia hybrida* (V30) expresses only one member of the chalcone synthase multigene family. Nucl. Acids Res. **14:** 5229-5239.

Koes, R.E., C.E. Spelt, P.J.M. van den Elzen, and J.N.M. Mol. 1989. Cloning and molecular characterization of the chalcone synthase multigene family of *Petunia hybrida*. Gene **81:** 245-257.

Kreuzaler, F., H. Ragg, E. Fautz, D.N. Kuhn, and K. Hahlbrock. 1983. UV-induction of chalcone synthase mRNA in cell suspension cultures of *Petroselinum hortense*. Proc. Natl. Acad. Sci. USA **80:** 2591-2593.

Metzlaff, M., M. O'Dell, R. Hellens, and R.B. Flavell. 2000. Developmental and transgene regulated nuclear processing of primary transcripts of *chalcone synthase A* in petunia. Plant J. **23:** 63-72.

Mol, J., E. Grotewold, and R. Koes. 1998. How genes paint flowers and seeds. Trends Plant Sci. **3:** 212-217.

Muir, S.R., G.J. Collins, S. Robinson, S. Hughes, A. Bovy, C.H. Ric De Vos, A.J. van Tunen, and M.E. Verhoeyen. 2001. Overexpression of petunia chalcone isomerase in tomato results in fruit containing dramatically increase levels of flavonols. Nat. Biotechnol. **19:** 470-474.

Napoli, C.A., D. Fahy, H.Y. Wang, and L.P. Taylor. 1999. *white anther*: a petunia mutant that abolishes pollen flavonol accumulation, induces male sterility, and is complemented by a chalcone synthase transgene. Plant Physiol. **120:** 615-622.

Napoli, C., C. Lemieux, and R. Jorgensen. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible cosuppression of homologous genes *in trans*. Plant Cell **2:** 279-289.

Oud, J.S.N., H. Schneiders, A.J. Kool, and M.Q.J.M. van Grinsven. 1995. Breeding of transgenic orange *Petunia hybrida* varieties. Euphytica **85:** 403-409.

Quattrocchio, F., J.F. Wing, H.T.C. Leppen, J.N.M. Mol, and R.E. Koes. 1993. Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. Plant Cell **5:** 1497-1512.

Schijlen, E.G.W.M., C.H. Ric de Vos, A.J. van Tunen, and A.G. Bovy. 2004. Modification of flavonoid biosynthesis in crop plants. Phytochemistry **65:** 2631-2648.

Seigler, D.S. 1998. Plant Secondary Metabolism. Kluwer Academic, Boston.

Suzuki, K.I., H.M. Xue, Y. Tanaka, Y. Fukui, M. Fukuchi-Mizutani, Y. Murakami, Y. Katsumoto, S. Tsuda, and T. Kusumi. 2000. Flower color modifications of *Torenia hybrida* by cosuppression of anthocyanin biosynthesis genes. Mol. Breed. **6:** 239-246.

Tinland, B. 1996. The integration of T-DNA into plant genomes. Trends Plant Sci. **1:** 178-184.

To, K.Y., D.F. Suen, and S.C.G. Chen. 1999. Molecular characterization of ribulose-1,5-bisphosphate carboxylase/oxygenase activase in rice leaves. Planta **209:** 66-76.

Tuteja, J.H., S.J. Clough, W.C. Chen, and L.O. Vodkin. 2004. Tissue-specific gene silencing mediated by a naturally occurring chalcone synthase gene cluster in *Glycine max*. Plant Cell **16:** 819-835.

Van der Krol, A.R., P.E. Lenting, J. Veenstra, I.M. van der Meer, R.E. Koes, A.G.M. Gerats, J.N.M. Mol, and A.R. Stuitje. 1988. An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. Nature **333:** 866-869.

Van der Krol, A.R., L.A. Mur, M. Beld, J.N.M. Mol, and A.R. Stuitje. 1990. Flavonoid genes in petunia: addition of a limited number of gene copies may lead to suppression of gene expression. Plant Cell **2:** 291-299.

Vaucheret, H., C. Béclin, T. Elmayan, F. Feuerbach, C. Godon, J-B. Morel, P. Mourrain, J-C. Palauqui, and S. Vernhettes. 1998. Transgene-induced gene silencing in plants. Plant J. **16:** 651-659.

Vaucheret, H., C. Béclin, and M. Fagard. 2001. Post-transcriptional gene silencing in plants. J Cell Sci. **114:** 3083-3091.

Wang, H.M. and K.Y. To. 2004. *Agrobacterium*-mediated transformation in the high-value medicinal plant *Echinacea purpurea*. Plant Sci. **166:** 1087-1096.

Wienand, U., U. Weydemann, U. Niesbach-Klosgen, P.A. Peterson, and H. Saedler. 1986. Molecular cloning of the *c2* locus of *Zea mays*, the gene coding for chalcone synthase. Mol. Gen. Genet. **203:** 202-207.

Winkel-Shirley, B. 2001. Flavonoid biosynthesis: a colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. **126:** 485-493.

Winkel-Shirley, B. 2002. Biosynthesis of flavonoids and effects of stress. Curr. Opin. Plant Biol. **5:** 218-223.

利用矮牽牛苯基苯乙烯酮合成酶對轉殖菸草苯基苯乙烯酮合成酶的共同抑制作用以產生白色花朵

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苯基苯乙烯酮合成酶（chalcone synthase，簡稱 *Chs*；EC 2.3.1.74）是花色素 (anthocyanin) 生合成路徑的關鍵酵素。欲了解花朵顏色的分子調控機制，我們利用農桿菌 (*Agrobacterium*) 轉殖基因的方法，將鑲嵌質體含 CaMV 35S 啟動子及矮牽牛 *chsA* cDNA 轉殖至菸草。所獲得轉殖菸草中，4 株轉殖株產生白色花朵，3 株轉殖株產生與野生型植株相同的粉紅色花朵。薄層色層分析的結果顯示，所有白色花朵的轉殖株皆缺乏花青素 (cyanidin)。北方氏墨點分析法顯示，白色花朵轉殖株內 *chs* mRNA 的總量大幅下降；相反的，花色屬粉紅色的轉殖株，*chs* mRNA 的總量卻顯著增加。RT-PCT 分析結果顯示，花色屬白色的轉殖株，其內生型菸草 *chs* mRNA 的相對量少於轉殖矮牽牛 *chs* mRNA。此外，植物/T-DNA 接合序列分析亦排除產生白色花色的原因是由於 T-DNA 插入植物色素合成相關基因導致該基因喪失活性的可能性。綜合而言，本研究提供了廣泛的實驗證據，說明菸草 *chs* 基因的共同抑制作用是可以透過別種植物如矮牽牛的相似基因而發揮作用，導致轉殖菸草產生白色花朵；並清楚顯示 *chs* mRNA 表現量、花青素含量及轉殖菸草花朵顏色的關聯性。

關鍵詞：花色素；苯基苯乙烯酮合成酶；共同抑制作用；花色之修飾；*Nicotiana tabacum*；轉殖植物；轉殖基因導致的基因靜默。