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Flavonoid components and flower color change in transgenic tobacco plants by suppression of chalcone isomerase gene

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Abstract **A cDNA encoding chalcone isomerase (CHI) was isolated from the petals of *Nicotiana tabacum* and the effect of its suppression on flavonoid biosynthesis was analyzed in transgenic tobacco plants. *CHI*-suppression by RNA interference (RNAi) showed reduced pigmentation and change of flavonoid components in flower petals. The plants also accumulated high levels of chalcone in pollen, showing a yellow coloration. Our results first demonstrated that suppression of *CHI* by genetic transformation is possible in higher plants. This suggests that CHI plays a major part in the cyclization reaction from chalcone to flavanone, and that spontaneous reactions are few, if any, in tobacco plants.**

Keywords: Chalcone isomerase; Flavonoid biosynthesis; Flower color; RNAi; *Nicotiana tabacum*

1. Introduction

Since the pioneering study on the creation of orange-flowered petunias by Meyer et al. [1], genetic engineering of flower colors in ornamental plants has been reported in many plant species. For commercial purposes, delphinidin-producing violet carnations have been developed by expression of heterologous flavonoid-3′,5′-hydroxylase (F3′,5′H) by the Florigene Ltd. (Australia) and Suntory Ltd. (Japan). On the other hand, downregulating anthocyanin biosynthetic genes such as chalcone synthase (CHS) with antisense or co-suppression techniques also resulted in a successful alternation of flower colors in many plant species [2,3]. For the purpose of producing newly colored flowers, many genes related to flavonoid biosynthesis have been identified, and transformation studies using various plant species are actively performed.

Chalcone isomerase (CHI, EC 5.5.1.6) is an enzyme in the flavonoid biosynthetic pathway in plants, which catalyzes the cyclization of chalcone into flavanone in the cytoplasm of plant cells. Overexpression of petunia *CHI* has been reported to increase flavonol contents in tomato fruit [4]. On the other hand, some plant species such as carnation, China aster and cyclamen, are known to accumulate chalcones, and show yellow pigmentation in the flowers by a reduction in CHI activity [5–7]. Molecular analyses has shown that disruption of *CHI* and dihydroflavonol 4-reductase (*DFR*) caused by transposon insertions resulted in yellow flowers in carnation [8]. Therefore, the lack of *CHI* activity seems essential for formation of yellow-flowered carnations. Petunia *po* mutant lines that have lost chiA promoter activity in anthers are also known to have yellow or greenish pollen [9]. Inactivation of CHI also results in a gold bulb color in an onion mutant by the accumulation of chalcone derivatives including a yellow pigment [10]. Therefore, it will be a prerequisite to inhibit CHI activity to produce yellow-flowered plants by pigmentation of chalcone or chalcone derivatives. However, there are no published studies on the suppression of any *CHI*, for the purpose of not only alternation of flower color, but also other objectives by genetic transformation. Actually, several attempts to suppress *CHI* have failed so far in lisianthus and petunia [11,12]. Though the reason is not fully understood, spontaneous conversion of chalcone to flavanone in vivo is speculated. The reaction mechanism of CHI has been recently studied in vitro using recombinant alfalfa CHI expressed in *Escherichia coli* [13,14]. However, it is still unknown if spontaneous cyclization of chalcone is a significant in vivo reaction in all plants. Therefore, to reveal whether *CHI* can or cannot be suppressed by genetic transformations in higher plants, we attempted to suppress *CHI* in a model plant tobacco by using the RNA interference (RNAi) technique. Here, we report the effects of *CHI* suppression in transgenic tobacco plants. The results showed a change of flavonoid components and colors, both in petals and pollen in transgenic tobacco plants. This is the first report that demonstrates the change of flower color by artificial *CHI* suppression by transgenic interference in higher plants.

2. Materials and methods

2.1. Isolation of CHI gene from tobacco petal

Total RNA was isolated from the petal of *Nicotiana tabacum* L. cv. SR1 and cDNAs were synthesized by the Takara RNA PCR Kit (AMV) Ver. 2.1 (Takara, Tokyo). To obtain a *CHI* gene, the partial fragment was amplified with degenerated primers obtained from conserved amino acid sequences of several plants including petunia, vitis, carnation and morning glory (forward primer: 5′-GGN AAR TTY RTN AAR TTY AC-3′; reverse primer: 5′-TTY TCR AAN GGN CCN GTN AC-3′). Ca. 150 bp of the amplified fragment were subcloned into TOPO TA cloning kit for sequencing (Invitrogen, CA), and subsequently sequenced. To further determine 3′-ends of sequence

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Abbreviations: CHI, chalcone isomerase; HPLC, high performance liquid chromatography; RNAi, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction

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of the *CHI* gene, rapid amplification of 3′-cDNA ends (3′RACE) was performed with a primer set as follows: 5′-CAG AGG AGT TGG CTA ATT CAC TCG-3′ and M13–M4. Thereafter, we obtained 875 bp of partial sequence of *CHI* cDNA from tobacco. 5′-RACE (5′ rapid amplification of cDNA ends) was performed to determine the full-length cDNA sequence using 5′RACE system version 2.0 (Invitrogen) according to the manufacturer's instructions. The 5′ end fragments were amplified using 5′-GACACTCTTTCGGCGATACTACAC-3′, then subcloned and sequenced as described above.

2.2. Southern blot analysis of endogenous CHI gene in tobacco plant

Total genomic DNA was isolated from 1 g leaf sample using Nucleon PhytoPure (Amersham Biosciences, NJ). Ten micrograms of genomic DNA digested by *Eco*RI or *Hin*dIII restriction enzymes (Takara) were separated on 0.6 % agarose gel and then transferred to a Nytran N membrane (Schleicher & Schuell, Germany). Probes for *NtCHI1* were prepared with the polymerase chain reaction (PCR)-DIG Probe Synthesis Kit (Roche Diagnostics, Germany) using primer sets for binary vector construction (see below). The hybridization and detection were performed as described previously [15].

2.3. Plasmid construction and tobacco transformation

The 543 bp fragment of *NtCHI1* containing coding and 3′UTR regions was amplified by the primer set as follows: 5′-TCT AGA TTG AGA AAT TCA CCC GAG TG-3′ (the underlined part was a *Xba*-I recognized sequence), and 5′-AGA TCT AGG CTC AGT TGA CAA AGG AG-3′ (the underlined part was a *Bgl*-II recognized sequence), and then subcloned into pCR2.1 TA cloning vector (Invitrogen). Each fragment in the sense and antisense orientations, which connected to the first intron of caster bean catalase [16] as a linker, was driven by the cauliflower mosaic virus 35S promoter. The expression cassette was inserted into a binary vector harboring hygromycin resistant gene (*hpt*) to produce plasmid pEBisHR-35SintNtCHIir (Supplementary Fig. S1). The construct was transformed into *Agrobacterium tumefaciens* EHA105 and used. Tobacco plants that were aseptically grown from seeds for about one month were transformed via an *A. tumefaciens*-mediated leaf disc procedure [17], and selected using 30 mg/l of hygromycin B. After rooting and acclimatization, the regenerated plants were grown in a greenhouse to set seeds by self-pollination. Transgenic T_1 plant lines selected on 30 mg/l hygromycin-containing medium were transferred to soil, and then used for further analyses.

2.4. Anthocyanin and chalcone analysis

To measure anthocyanin amounts in the petals in transgenic tobacco lines, anthocyanin compounds were extracted by methanol containing 1% hydrochloric acid. Anthocyanin concentrations were estimated by measuring the absorbance of extracts at 530 nm using a spectrometer.

To investigate flavonoid compounds accumulating in the petals and pollen of transgenic tobacco plants, flavonoid compounds were extracted from petals and anthers of transgenic plants by methanol, and high performance liquid chromatography (HPLC) analysis was performed as described previously [15]. The absorbance was monitored at 360 nm and obtained peaks were also checked with a photodiode array detector; 2′,4,4′,6′-tetrahydroxychalcone was prepared from naringenin as described [18] and used as the standard. The samples extracted from *CHI*-suppressed tobacco anthers were also subjected to HPLC under co-separation conditions with the standard.

2.5. Northern blot analysis

Total RNAs were isolated from petals of wild type tobacco plants in four different developmental stages, and transgenic tobacco petals just before anthesis, respectively. Five micrograms of total RNAs were subjected to Northern blot analysis using the probe described in Section 2.2. Hybridization and detection were performed as described previously [15].

Small interfering RNA (siRNA) of *CHI* was detected according to Goto et al. [19]. The low molecular weight RNAs were separated from 20 μg total RNAs using the RNA cleanup protocol of RNeasy mini kit (QIAGEN, Japan). siRNAs separated on 15% polyacrylamide gel electrophoresis were transferred to Hybond-N+ membrane (Amersham Biosciences), and hybridized by DIG-PCR probe described above.

3. Results

3.1. Cloning and characterization of CHI cDNA from tobacco petal

A 147 bp fragment was amplified from tobacco petal cDNAs by degenerate PCR. By 3′ and 5′ RACE method, a 1008 bp length of tobacco *CHI* cDNA was obtained and termed as *NtCHI1* (Accession No. AB213651). The deduced amino acid sequence of *NtCHI1* exhibited 80% and 77% identities with CHIA (P11650) and CHIB (P11651) of the petunia, respectively (Supplementary Fig. S2). NtCHI also showed a relatively high homology to CHIs of several higher plants, including vitis (CAA53577), autumn-olive (AAC16013), carnation (CAA91931) and citrus (BAA36552). Northern blot analysis showed the *NtCHI1* transcripts were constantly accumulated at high levels throughout flower development (Fig. 1A). Southern blot analysis suggested the presence of at least two copies of *CHI* homologues in the tobacco genome (Fig. 1B).

3.2. Production of CHI-suppressed tobacco transformants by RNAi

A binary vector expressing double-strands RNA of *NtCHI1* was transformed into tobacco plants. Twelve independent transgenic tobacco plants were produced and grown in a greenhouse. Alternation of colors in flower petals and pollen was observed in several transgenic plant lines. To confirm the phenotype in detail, the seeds were collected after self-pollination and seven independent T_1 transgenic plant lines were subjected to further analysis.

3.3. Phenotypes and expression analysis of CHI-suppressed tobacco transformants

T_1 transgenic tobacco plants were analyzed for accumulations of anthocyanin and *NtCHI1* mRNA in the petals (Fig. 2). All transgenic plants showed up to 25% reduction in anthocyanin content of the wild type. Northern blot analysis showed the decreased levels of *NtCHI1* mRNA accumulation in all transformants, and no clear signals were detected in some plant lines. The siRNA analysis in the petals also showed the presence of *NtCHI1*-derived small RNA, except for line nos.

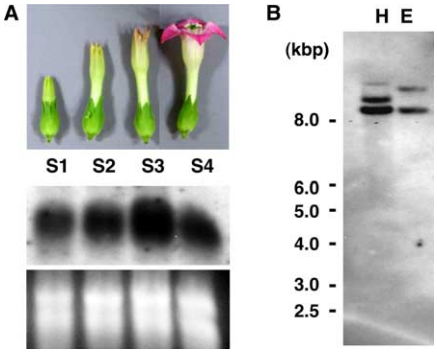

Fig. 1. Expression profile and copy number of *NtCHI1* gene in tobacco plant. (A) Four flower developmental stages defined in this study. Five microgram of RNAs isolated from the petals at each flower developmental stage (S1–S4) were separated, and hybridized with *NtCHI1* probe. (B) Five micrograms of genomic DNAs digested by *Hin*dIII (H) or *Eco*RI (E) restriction enzymes were separated and hybridized with the *NtCHI1* probe.

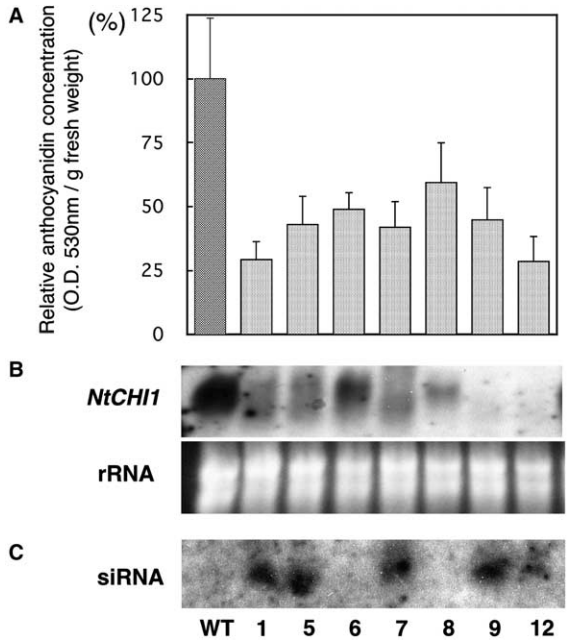

Fig. 2. Anthocyanin concentrations and suppression of *NtCHI1* transcripts in transgenic tobacco plants. (A) Anthocyanin was extracted from the petals of an untransformed control plant (WT) and seven transgenic T_1 plants (line nos. 1–12), and the absorbance of 530 nm was measured using a spectrometer. Data indicate average values ± SDs of 3–10 replicates. (B) Total RNAs isolated from the petals at stage 3 were subjected to Northern blot analysis. (C) siRNAs purified from the total RNAs were separated on PAGE, and hybridized with *NtCHI1* probe as described in Section 2.

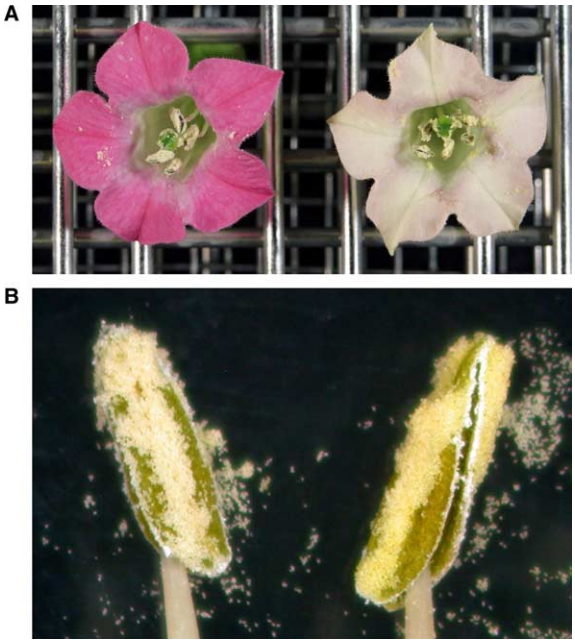

Fig. 3. Phenotype of *CHI*-suppressed transgenic tobacco plant. Flowers (A) and dehiscence anthers (B) of untransformed control plants (left) and transgenic plant line no. 1 (right).

6 and 8, indicating the silencing was due to post-transcriptional gene silencing (PTGS). The presence of siRNA correlated well with the decreased levels of the *NtCHI1* transcripts (Fig. 2B and C).

A typical flower with reduced pigmentation is shown in Fig. 3. Line nos. 1 and 12 showed almost white flowers, and the remainder of the transgenic lines showed pale pink flowers. This also coincided well with anthocyanin accumulation levels as determined above. In addition, pollen of transgenic tobacco plant line nos. 1 and 12 were yellow in color compared with the white color of the wild type.

3.4. HPLC analysis of petals and anthers of CHI-suppressed tobacco transformants

Line no. 1, which showed clear reduction of anthocyanin and change of petal and pollen color, was analyzed by HPLC (Fig. 4). The results showed that two additional peaks were observed both in petals and pollen of the transgenic plants. In the petals, an additional new peak 1 and a small peak 2 were detected compared with the wild type (Fig. 4A). On the other hand, a major peak 2 and a small additional peak 1 with the same retention times as the new peaks in petals of transgenic plants were detected in pollen of transgenic plants (Fig. 4B). When each area was calculated on a dry weight basis, the peaks 1 and 2 in transgenic pollen were about two times and 190 times larger than those in transgenic petals, respectively. The additional major peaks in transgenic tobacco plants were checked with a photodiode array detector and the typical results are shown in Fig. 5. The peak 1 in transgenic petals showed the absorbance profile almost identical to the standard

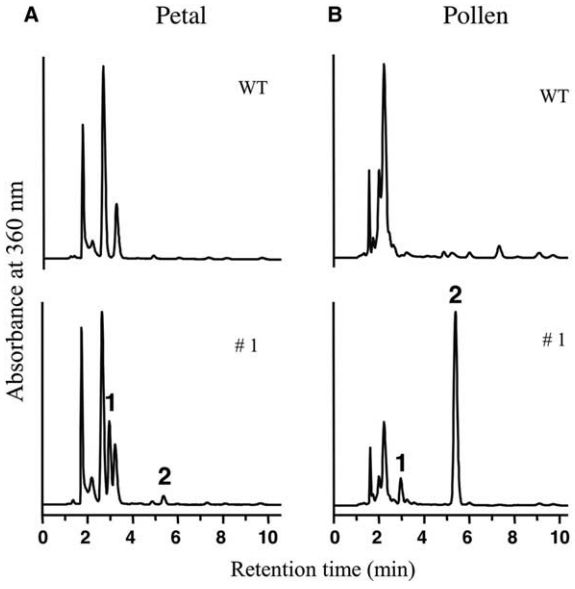

Fig. 4. HPLC profiles of the petals and pollen of *CHI*-suppressed transgenic tobacco plant. The flavonoid compounds extracted from petals (A) and pollen (B) of untransformed control plants (WT) and transgenic plant line no. 1 (#1) were subjected to HPLC analysis. A peak 1 indicates an uncharacterized compound and a peak 2 indicates a compound 2′,4,4′,6′-tetrahydroxychalcone.

2′,4,4′,6′-tetrahydroxychalcone. The peak 2 in transgenic pollen showed two maximum absorbances about 300 and 360 nm. Notably, maximum absorbance at 360 nm was detected only in transgenic pollen, showing chalcone accumulation. Co-separation experiments with a naringenin chalcone standard supported that the peak 2 in transgenic pollen corresponds to 2′,4,4′,6′-tetrahydroxychalcone (Fig. S3). These

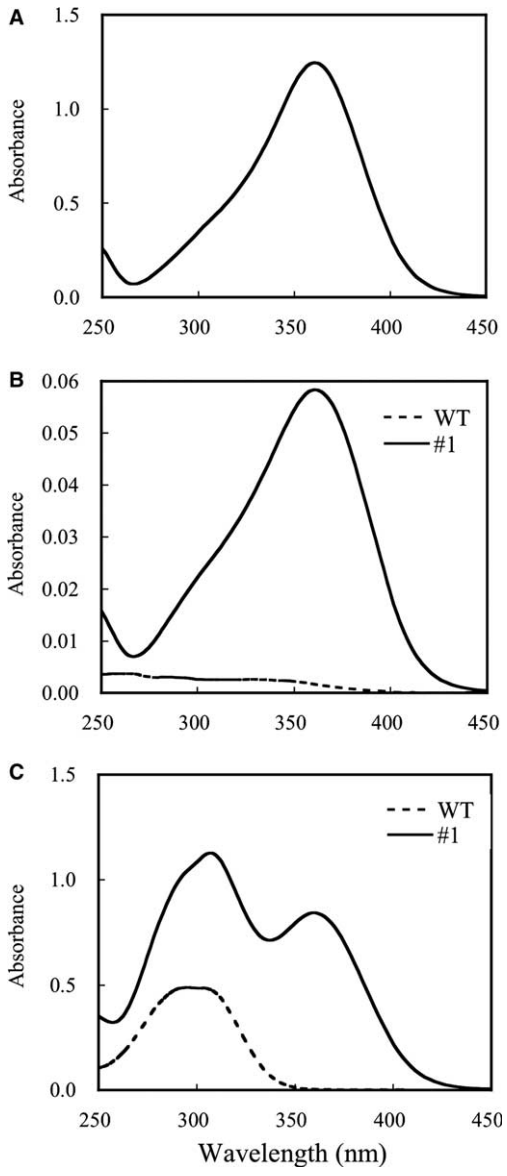

Fig. 5. Absorbance spectra of peaks 1 and 2 of the transgenic and wild type petals and pollen. Each peak separated by HPLC as in Fig. 4 was measured by a photodiode array detector: (A) a standard naringenin chalcone (2′,4,4′,6′-tetrahydroxychalcone); (B) peak 1 of petals; (C) peak 2 of pollen.

results suggested the additional peaks contained chalcone and probably chalcone derivatives.

4. Discussion

Our results clearly demonstrated that suppression of *CHI* could be achieved in transgenic tobacco plants by RNAi transformation, and the alternation of flavonoid components and colors occurred in both petals and pollen. Compared with antisense or co-suppression techniques [3], RNAi technology is considered to be more effective. A strong inhibition of the target gene expression has been shown in many other studies [20–22]. In fact, siRNAs detection in our transgenic tobacco plants confirmed that the suppression was really induced by the sequence-specific mRNA degradation during the process of RNAi [23].

In *CHI*-suppressed tobacco plants, high levels of chalcone only accumulated in pollen, but not in petals. Some compounds that belong to the chalcone group are known to be toxic and inhibit cell growth, and induction of apoptosis against animal cells have been studied [24–26]. Chalcones have been also reported to inhibit cytochrome P4501A activity of rat in vitro [27]. Although the suppression of *CHI* will first cause accumulation of chalcone in cytoplasm, endogenous enzyme(s) appear to convert chalcone to atoxic compounds in petals for detoxification or degradation. Chalcone accumulation in petals may be attributed to an enzymatic reaction such as chalcone glucosylation prerequisite for transport into the vacuoles. Chalcone glucosyltransferases have recently been identified in carnation as enzymes to the glycosylate 2′ position of chalcone to synthesize chalcone 2′-glucoside [28]. Therefore, it is likely that endogenous unknown enzyme(s) catalyzes parts of the accumulated chalcone to chalcone derivatives (also in our transgenic tobacco plants). In fact, HPLC analysis of the petals showed a new peak that was never observed in untransformed control tobacco plants (Fig. 4), though the compound still remains to be identified. On the other hand, pollen of *CHI*-suppressed tobacco plants clearly showed the accumulation of high levels of chalcone aglycone and yellow color (Fig. 3), indicating that pollen cells lack such enzyme(s) for detoxification or degradation of chalcone, and the accumulated chalcone does not influence pollen development and viability. This is supported by the fact that the transgenic plants were fertile and set seeds normally. The alternative view is that different cellular environments in petals and pollen might contribute to yellow color pigmentation only in pollen. In lisianthus, it is proposed that yellow coloration in flowers can result from aggregation of flavonoid glycosides on a protein matrix in petal epidermal cell cytoplasm [29]. It is also probable that the chalcone extracted from the outer wall of pollen (the exine) might confer the yellow color. In the absence of CHI activity in anthers, petunia *po* mutant shows yellow or greenish pollen by accumulation of the substrate of CHI, although it can still form flavonols after the CHI step [9,30]. Maize cv. Black Mexican Sweet (BMS) cells with undetectable CHI activity can produce anthocyanins and 3-deoxy flavonoids, indicating that CHI is not required for flux through any of the flavonoid pathways [31]. It is also speculated that the reaction that catalyzes chalcone is different among plant species and also among cell types, and the intermediates or final accumulated products can be harmful against some plant species when CHI activity is substantially suppressed. This could explain the difficulty to obtain *CHI*-suppressed plants in particular plant species.

Since the transgenic tobacco plants did not show a complete white flower color, there are two possibilities: (1) the suppression of *NtCHI1* by RNAi strategy was incomplete and the remaining low CHI activity contributed for anthocyanin accumulation, (2) a spontaneous reaction of chalcone into flavanone occurred, even with complete suppression of *CHI*. The loss of functional analysis for all *CHI* loci in tobacco plants will confer a definitive answer in future studies.

In conclusion, our results clearly demonstrated that CHI plays a major role in the cyclization reaction from chalcone to flavanone in vivo using transgenic tobacco plants. The *CHI*-suppressed tobacco plants will be used to search for enzymes that catalyze chalcone as a substrate and for

fundamental studies of genetic engineering in yellow-colored flowers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.09.073.

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