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Simultaneous post-transcriptional gene silencing of two different chalcone synthase genes resulting in pure white flowers in the octoploid dahlia

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Abstract

Garden dahlia (Dahlia variabilis) are autoallooctoploids with redundant genes producing wide color variations in flowers. There are no pure white dahlia cultivars, despite its long breeding history. However, the white areas of bicolor flower petals appear to be pure white. The objective of this experiment was to elucidate the mechanism by which the pure white color is expressed in the petals of some bicolor cultivars.

A pigment analysis showed that no flavonoid derivatives were detected in the white areas of petals in a star-type cultivar ‘Yuino’ and the two seedling cultivars ‘OriW1’ and ‘OriW2’ borne from a red-white bicolor cultivar, ‘Orihime’, indicating that their white areas are pure white. Semi-quantitative RT-PCR showed that in the pure white areas, transcripts of two chalcone synthases (CHSs), DvCHS1 and DvCHS2 which share 69% nucleotide similarity with each other, were barely detected. Premature mRNA of DvCHS1 and DvCHS2 were detected, indicating that these two CHS genes are silenced post-transcriptionally. RNA gel blot analysis revealed that small interfering RNAs (siRNAs) derived from CHSs were produced in these pure white areas. By high-throughput sequence analysis of small RNAs in the pure white areas with no mismatch acceptance, small RNAs were mapped to two alleles of DvCHS1 and two alleles of DvCHS2 expressed in ‘Yuino’ petals. Therefore, we concluded that simultaneous siRNA-mediated post-transcriptional gene silencing of redundant CHS genes results in the appearance of pure white color in dahlia.

Key words: CHS, Dahlia variabilis, PTGS, pure white flower, siRNA

Abbreviations

3GT          Anthocyanidin 3-glucosyltransferase
ANS          Anthocyanidin synthase
bHLH         Basic helix-loop-helix
CHI          Chalcone isomerase
CHS          Chalcone synthase
DFR          Dihydroflavonol 4-reductase
F3H          Flavanone 3-hydroxylase
FLS          Flavonol synthase
FNS          Flavone synthase
HPLC         High performance liquid chromatography
ORF          Open reading frame
PTGS         Post-transcriptional gene silencing
siRNA        Short interfering RNA
SNPs         Single nucleotide polymorphisms
TLC          Thin layer chromatography
TGS          Transcriptional gene silencing
WDR          WD40 repeats
**Introduction**

In garden dahlias (*Dahlia variabilis*), as many as 50,000 cultivars with various colors, shapes, and sizes of inflorescences have been bred in the past 100 years (McClaren 2009). Especially striking are the flower color variations, including purple of anthocyanins, yellow of chalcones and/or aurones, and red to orange resulting from the coexistence of both the pigments. Garden dahlias are octoploid (2n = 8x = 64) resulting from doubled allotetraploid (2n = 4x = 32) (Gatt et al. 1998); this feature has enabled the production of several cultivars. Although a great variety of flower colors has been produced, almost all commercially available white-flowered cultivars are ivory white. Many growers and researchers have attempted to breed pure white cultivars, which do not accumulate any pigments in the petals giving them a transparent look (Bate-Smith et al. 1955). There are many star type dahlia cultivars with partially pure white areas on the petals; however, completely pure white cultivars, which have a potentially large market for ceremonial use, cannot be found.

There are several studies on the differences in pigment composition and gene expression between pure white and ivory white flowers in horticultural crops (Spribille and Forkmann 1982; Onozaki et al. 1999; Mato et al. 2000). These studies have concluded that the termination step in the anthocyanin biosynthesis pathway is important for determining pure white or ivory white expression. Termination of the expression of early genes in anthocyanin biosynthesis results in pure white petals as petals then do not accumulate any flavonoid derivatives. Termination of late gene results in ivory white petals as the petals accumulate intermediate or derivative products, such as flavone in gentian plants (Nakatsuka et al. 2005; Nakatsuka et al. 2010) or flavonol in dianthus plants (Onozaki et al. 1999; Mato et al. 2000). Chalcone synthase (CHS) is the first enzyme in the biosynthesis of various flavonoid derivatives and metabolic products in plants, such as naringenin, flavones, and flavonols, as well as butein and aurone derivatives. The flowers of CHS mutants of *Antirrhinum majus* (Spribille and Forkmann 1982), *Ipomoea nil* (Hoshino et al. 2009), and *Matthiola incana* (Hemleben et al. 2004) contain no flavonoid derivatives in petals and express a pure white phenotype. However, CHS is known to belong to multigene family, and very few completely pure white flower cultivars are known.

On exposure to ammonia gas, the white petals of commercial white cultivars become yellow; however, the white areas of star-type dahlia cultivars remain white, suggesting that they do not contain any flavonoids. Thus, suppression of chalcone synthesis was expected in the white areas. However, dahlias have redundant CHS derived from their high polyploidy. We have isolated two different dahlia CHS, *DvCHS1* (AB576660) and *DvCHS2* (AB591825) that shared only 69% nucleotide similarity in their coding regions. All these CHS genes have conserved CHS active site residues (Ferrer et al. 1999; Ma et al. 2009) and the characteristic intron insertion site conserved in polyketide synthase (Zheng et al. 2001). In addition to this, 12 sequences of *DvCHS1* mRNA and two sequences of *DvCHS2* mRNA are expressed in petals of a bicolor cultivar ‘Michael J’ (Ohno et al., unpublished data). In the seed coats of yellow soybean (*Glycine max*), which has nine CHS, all CHS are simultaneously silenced by post-transcriptional gene silencing (PTGS) (Kurauchi et al. 2009; Tuteja et al. 2009). As an example of a more aggressive gene suppression process, PTGS, RNA interference and virus-induced gene silencing are known to suppress multigene family members and redundant genes simultaneously, for example, in polyploid species (Napoli et al. 1990; Van Der Krol et al. 1990; Lawrence and Pikaard 2003; Fukusaki et al. 2004; De Paoli et al. 2009; Jiang et al. 2011). PTGS of CHS producing pure white flowers is observed in several horticultural crops, such as star-type cultivars of *Petunia hybrida* (Metzlaff et al. 1997; Koseki et al.
2005). For these reasons, PTGS of CHS is expected to result in pure white areas of petals in dahlia. However, whether it is possible to silence two CHS sharing low identity simultaneously by PTGS has been unclear.

The object of this study was to confirm that all CHS expression in apparently pure white areas of petals are simultaneously silenced by PTGS. We used star-type cultivars and original cultivars, which produce flowers that appear pure white. First, we confirmed that the white areas of the star-type cultivars and original cultivars are pure white by pigment analysis. Second, we ascertained that CHS, and not other genes, are silenced in the white areas. Third, we analyzed small RNAs in the white areas by deep sequencing, and mapped them on all CHS isolated from a star-type cultivar ‘Yuino’. We have concluded with a discussion about pure white color expression in dahlias.

Materials and methods

Plant materials

Three star-type cultivars ‘Yuino’, ‘Matsuribayashi’, and ‘Kazusa-shiranami’ (Figs. 1a–c), two commercial white-flowered cultivars ‘Hakuyo’ and ‘Malcoms White’ (Figs. 1d, e), and two original white-flowered strains OriW1 (Fig. 1g) and OriW2 (Fig. 1i) were used. OriW1 and OriW2, seedlings selected from the natural crossing of ‘Orihime’ (Fig. 1f), were propagated by cuttings. They produce relatively stable, pure white flowers; however, they spontaneously produce flowers with white-colored and yellow- or red-colored petals, respectively (Figs. 1h, j). These colored petals were also used for the experiments. The rooted cuttings of all cultivars were transplanted to the Experimental Farm of Kyoto University (Kyoto, Japan) and the just-opened petals were collected during the flowering seasons.

Pigment analysis

High performance liquid chromatography (HPLC) (LC10A system, Shimadzu, Kyoto, Japan) was used to separate hydrolyzed pigments. Pigments were extracted from 1.0 g of fresh petals using 10% methanol-acetic acid solution (methanol:acetic acid = 9:1 v/v) for 24 h at 4°C. For star-type cultivars, harvested petals were separated into the colored and white areas using a razor blade. The 2N hydrochloric acid-extracted solutions of each cultivar were boiled for 2 h and used as crude aglycones. HPLC was conducted on an HPLC system with a C18 column (Nihon Waters K.K., Tokyo, Japan), and peaks were detected with a photodiode array detector. The detection wavelength was 350 nm for flavones, 380 nm for chalcones and aurones, and 530 nm for anthocyanidins. Eluant A was 1.5% phosphate dissolved in water and eluant B was 1.5% phosphate, 20% acetic acid, and 25% acetonitrile dissolved in water. The analysis period for each sample was 45 min and comprised 0 min with 20%, 40 min with 85%, and 20% with eluant B at a flow rate of 1 ml min⁻¹ at 40°C. As for standards, commercially available naringenin (Wako, Kyoto, Japan), apigenin (Wako), and luteolin (Wako) and thin layer chromatography (TLC) and HPLC-separated and -purified hydrolyzed cyanidin and pelargonidin from rose petals were used. To obtain hydrolyzed standards of butein, isoliquiritigenin, sulfuretin, and aurone, extracts from the orange petals of the dahlia strain HywR7R (photograph not shown) were separated by paper chromatography and each band was eluted with methanol. Each eluate was further dried and dissolved in a small amount of methanol. The color, Rf value, and maximum wavelength of the eluted compounds were measured
(U-2000A, Hitachi Ltd., Tokyo, Japan) and the compounds were determined by comparing the data with those of authentic butein (kindly supplied by Dr. Norio Saito) and previously reported data (Nordström and Swain 1956). TLC was performed on cellulose-coated plastic sheets (Merck Chemicals Japan, Tokyo, Japan) using two mobile phases: BAW (α-butanol: acetic acid: water = 4:1:2 v/v/v) and 30% acetic acid.

Total RNA extraction

To analyze the gene expression relating to the anthocyanin biosynthesis pathway, total RNA was extracted from just-opened petals using QuickGene RNA cultured Cell Kit S (Fujifilm, Tokyo, Japan) according to the manufacturer’s instructions with some modifications. For bicolor cultivars, the whole colored or white area of petals, separated using a razor blade, was used for the extraction. A 0.5-g sample of fresh petals powdered in liquid nitrogen was suspended in the lysis buffer of the cultured cell kit containing 0.3 U α-amylase (Sigma-Aldrich Japan, Osaka, Japan) and incubated for 3 min at room temperature to allow the degradation of the large amount of polysaccharides in the sample. After the amylase treatment, the solution was introduced into the equipped column, and 20 U of DNase (Wako) was added. RNA concentrations were standardized to 100 ng per μl using a spectrophotometer (Nano drop 1000; Thermo Fisher Scientific, Waltham, MA, USA).

Sequencing of DvCHS1 and DvCHS2 expressed in ‘Yuino’ petals

Total RNA extracted from bicolor ‘Yuino’ petals were reverse transcribed by ReverTra Ace (Toyobo, Ohtsu, Japan) using an oligo-(dT)20 primer. Reverse transcripts were amplified with Blend Taq polymerase (Toyobo) through the following steps: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were cloned into pTAC vectors using DynaExpress TA PCR Cloning Kit (BioDynamics Laboratory Inc., Tokyo, Japan) and 20 plasmids of each gene were sequenced. All sequence analyses were performed using a BigDye Terminator v 3.1 Cycle Sequencing Kit and a 3100 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA). Primers used for PCR are shown in Table 1.

Semi-quantitative RT-PCR

The reaction solution for reverse transcription (RT) composed of 8 μl of RT buffer (ReverTra Ace, Toyobo), 4 μl of 10 mM dNTPs, 2 μl of RNase inhibitor (Toyobo), 4 μl of oligo-(dT)20 (20 mM), and 2 μl of ReverTra Ace and was made up to 36 μl with ultrapure water. A 400-ng (4 μl) sample of template total RNA was used for the RT reaction which was conducted at 42°C for 30 min followed by 99°C for 5 min, and 1 μl of the RT reaction solution was used for PCR. The PCR solution composed of 0.1 μl of Blend Taq polymerase (Toyobo), 1.0 μl of 2 mM dNTPs, 1.0 μl of Blend Taq buffer, 0.1 μl of each primer (20 μM), and 1.0 μl of RT reaction solution and was made up to 10 μl with ultrapure water. The PCR reaction was as follows: 94°C for 2 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s, and finally 72°C for 10 min. A 5-μl sample of PCR solution was separated on 1% agarose gel and visualized using ethidium bromide. DvActin was used as an internal standard for the semi-quantitative RT-PCR. Primers used for the semi-quantitative RT-PCR are shown in Table 1.

To quantify the two different CHS separately, primers were designed for the cDNA sequences
that differed between \textit{DvCHS1} and \textit{DvCHS2}. We revealed two alleles each, \textit{DvCHS1-1} and \textit{DvCHS1-2} for \textit{DvCHS1}, and \textit{DvCHS2-1} and \textit{DvCHS2-2} for \textit{DvCHS2}, for each \textit{CHS} which were expressed in ‘Yuino’ petals. Consequently, the primers for quantification of \textit{DvCHS1} were designed from the homologous sequences of \textit{DvCHS1-1} and \textit{DvCHS1-2}, and primers for \textit{DvCHS2} were designed from the homologous sequences between \textit{DvCHS2-1} and \textit{DvCHS2-2}. Primers for \textit{chalcone isomerase (DvCHI)}, flavone 3-hydroxylase (DvF3H), and dihydroflavonol 4-reductase (DvDFR) (Table 1) were designed in identical regions among a number of previously determined 7, 11, and 10 different sequences, respectively. \textit{Anthocyanidin synthase (DvANS)} and \textit{anthocyanidin 3-glucosyltransferase (Dv3GT)} (Table 1) were identified in dahlias and primers were selected from our preliminary sequence data. To isolate cDNA clones of \textit{MYB} genes or WD40 repeats (WDR) genes, we screened a cDNA library (Suzuki et al. 2002) assembled from dahlia petals kindly provided by Dr. Yoshikazu Tanaka (Suntory, Osaka, Japan). Basic helix-loop-helix (bHLH) genes were isolated with degenerate primers and rapid amplification of cDNA ends. Primers were selected from several sequences among different clones (Table 1). Partial sequences of flavone synthase (DvFNS) and flavonol synthase (DvFLS) were isolated with degenerate primers (data not shown).

Real-time RT-PCR

For the real-time RT-PCR of \textit{DvCHS1} and \textit{DvCHS2}, first-strand cDNA was added to the reaction solution of SYBR Premix Ex TaqII (Takara Bio Inc., Otsu, Japan) according to the manufacturer’s instructions and analyzed using a 7900HT Fast Real-Time PCR System (Applied Biosystems). The real-time RT-PCR reaction was conducted as follows: 95°C for 30s followed by 40 cycles of 95°C for 5 s and 60°C for 15 s; the single target product proliferation was checked using dissociation curves. For the relative quantification of premature mRNA, CHS1-R1142 or CHS2-Full-R primer (Table 2) was used instead of a oligo-(dT)$_{20}$ primer to synthesize first-strand cDNA. The primer set for premature \textit{DvCHS1} mRNA was designed from a number of genomic sequences of abundantly expressed \textit{DvCHS1} alleles, as previously determined, and the primer set for premature \textit{DvCHS2} mRNA was designed from identical regions of two different genome sequences of \textit{DvCHS2} alleles. The intron lengths were 708bp and 822bp for \textit{DvCHS1} and 96bp and 106bp for \textit{DvCHS2} (Ohno et al. unpublished data). \textit{DvActin} was used as an internal standard.

Feeding experiments

To confirm the stop position of the anthocyanin biosynthesis pathway, naringenin (product of CHI) or taxifolin (product of F3H) was fed to ‘Hakuyo’ petals or white areas of the petals of ‘Yuino’, OriW1, and OriW2. A sample of 500 mg of naringenin (Wako) or taxifolin (Wako) was dissolved in a small amount of ethanol and made up to 1 ml. Just-opened petals cut vertically to the midrib in the middle portion were each immersed in the solution and incubated at 20°C overnight under dark conditions. The color changes of the immersed petals were determined by their appearances, and the pigments of the reddish petals were extracted and analyzed by HPLC according to the method described above.

Determination and mapping of short interfering RNA of \textit{CHS}

Short interfering RNA (siRNA) was detected following the method of (Hamilton and Baulcombe 1999) with some modifications. Crude total RNA containing siRNA was extracted from 0.5 g of just-opened
petals with 1 ml Trizol reagent (Invitrogen, Carlsbad, CA, USA). A 20-μg RNA sample was separated on 30% acrylamide gel in 0.5 x TBE buffer and subsequently electroblotted to a Hybond N+ membrane (GE Healthcare Japan, Tokyo, Japan) in 0.5% TBE buffer. Digoxigenin-labeled RNA probe was synthesized using T7 RNA polymerase (F. Hoffmann-La Roche AG, Basel, Switzerland) by in vitro transcription of 3’ digested pTAC-1 vector carrying the full length of DvCHS1 in the antisense orientation. For random digestion of the 1,500 bp dig-labeled RNA probe into sections approximately 150 bp long, 15 μg synthesized RNA probe was mixed with an equal volume of carbonic acid buffer (120 mM Na2CO3 and 80 mM NaHCO3; pH 10.2) and incubated at 60°C for 54 min. The reaction was stopped by adding 10% acetic acid to the solution to a final concentration of 0.5%. After EtOH precipitation, the probe was dissolved in ultrapure water, hybridized to the membrane at 42°C overnight, and the membrane was washed with high- and low-stringency buffers at 42°C and 25°C, respectively. Detection was conducted with CDP Star (GE Healthcare Japan) and the fluorescence image was obtained using a LAS-3000 Mini (Fujifilm).

For deep sequencing analysis of small RNAs accumulating in the white areas of ‘Yuino’, small RNAs were extracted from 1.0 g of fresh pure white petals using a MirVana miRNA Isolation Kit (Applied Biosystems) according to the manufacturer’s instructions and sequenced using an Illumina Genome Analyzer (Illumina Inc., San Diego, CA, USA). In this experiment, two alleles of CHS genes were determined in each subfamily, DvCHS1-1 and DvCHS1-2 as DvCHS1 and DvCHS2-1 and DvCHS2-2 as DvCHS2, in the petals of ‘Yuino’. These two alleles of CHS genes could be identified by 19 single nucleotide polymorphisms (SNPs) and 39 SNPs, respectively, in their full length mRNAs (Supplementary data Table S1). The nucleotide similarity between the two CHS1 genes is 98% and that between the two CHS2 genes is 96%. Sequenced small RNAs of 18–32 nt were mapped in each CHS gene with no mismatch.

Accession numbers

The accession numbers of genes used in this study are as follows: DvCHS1-1 (AB576660), DvCHS1-2 (AB576661), DvCHS2-1 (AB591825), DvCHS2-2 (AB591826), DvCHS1 genome region (AB621919), DvCHS2 genome region (AB621920), DvCHI (AB591827), DvF3H (AB591828), DvDFR (AB591829), DvANS (AB591830), DvMYB1 (AB601003), DvMYB2 (AB601004), DvR3MYB (AB621921), DvIVS (AB601005), DvDEL (AB601006), DvWDR1 (AB601007), DvWDR2 (AB601008), and DvActin (AB621922).

Results

Pigment determinations

Flavones, such as apigenin and luteolin derivatives, were detected in the petals of the commercial white-flowered cultivars ‘Hakuyo’ and ‘Malcoms White’ (Figs. 1d, e), whereas anthocyanidin, chalcone and aurone derivatives remained undetected (Table 3). The colored areas of star-type cultivars ‘Yuino’, ‘Matsuribayashi’, and ‘Kazusa-shiranami’ (Figs. 1a–c) contained anthocyanidin and flavone derivatives. Among these, the chalcone derivatives and aurone derivatives were detected in ‘Yuino’ and ‘Matsuribayashi’. However, no flavonoid derivatives were detected in the white areas of these two
star-type cultivars (Table 3). Traces of flavone derivatives were detected in the white area of ‘Kazusa-shiranami’, which could be contamination from the colored margin (Table 3). In the white areas of OriW1 and OriW2, no flavonoid compound was detected (Table 3). However, flavonoid derivatives were detected in the spontaneously produced colored petals in the inflorescences of these two strains (Fig. 1h, j); chalcone, aurone, and flavone derivatives were detected in the yellow petals of OriW1 and anthocyanidin, chalcone, aurone, and flavone derivatives in the red petals of OriW2 (Table 3). These results showed that the white area of star-type cultivars, OriW1 and OriW2 are pure white whereas white petals of commercial white cultivars are not.

Semi-quantitative RT-PCR and feeding experiments

We analyzed the expression of the genes involved in flavonoid biosynthesis, including nine structural genes (DvCHS1, DvCHS2, DvCHI, DvFNS, DvFLS, DvF3H, DvDFR, DvANS, and Dv3GT) and seven regulatory genes (DvMYB1, DvMYB2, DvR3MYB, DvIVS, DvDEL, DvWDR1, and DvWDR2), by RT-PCR in ‘Hakuyo’, ‘Malcoms White’, ‘Yuino’, OriW1 and OriW2 (Fig. 2). In the commercial white cultivars, little or no expressions of DvCHS1, DvF3H, DvDFR, and DvANS were detected (Fig. 2). Of the seven regulatory genes, only DvIVS, a bHLH transcription factor orthologous to TT8 of Arabidopsis thaliana (Nesi et al. 2000), An1 of Petunia hybrida (Quattrocchio et al. 1993; Spelt et al. 2000; Spelt et al. 2002), IpIVS of Ipomoea tricolor (Park et al. 2004), and IpIVS of Ipomoea purpurea (Park et al. 2007) were not expressed (Fig. 2). DvIVS expression seemed to correspond with those of DvCHS1, DvF3H, DvDFR, and DvANS indicating that the expressions of these structural genes are activated by DvIVS. Transcripts of other genes including DvCHS2 were detected in these commercial white cultivars (Fig. 2). In both the colored and pure white areas of ‘Yuino’ and OriW2, transcripts of all the structural genes were detected. But the signals of the RT-PCR products of DvCHS1 and DvCHS2 in the pure white areas were weaker than those in the colored areas (Fig. 2). Although traces of DvF3H and DvANS in the pure white areas were observed in both the spontaneously produced yellow areas and the pure white areas of OriW1, suppressions of DvCHS1, DvF3H, DvDFR, DvANS, and DvIVS were also observed. In addition, lower expression levels of DvCHS2 were observed in the pure white areas of OriW1 (Fig. 2).

To confirm that only CHS was suppressed in the pure white areas, we performed a chemical feeding experiment using naringenin and taxifolin, which are synthesized by CHI and F3H, respectively. Since chalcone (4,2′,4′,6′-tetrahydroxylchalcone) synthesized by CHS spontaneously converts to naringenin in solutions without CHI, we used naringenin instead of chalcone (4,2′,4′,6′-tetrahydroxylchalcone). If the anthocyanin synthesis pathway below CHS is normal, petals turn to red by synthesized anthocyanins. As expected, the pure white areas of ‘Yuino’ and OriW2 turned red by the feeding treatment (Supplementary data Fig. S1). HPLC showed the red pigments to be cyanidin and pelargonidin derivatives (data not shown). However, anthocyanin production in the fed portion was not observed after naringenin and taxifolin feeding treatments in ‘Hakuyo’ and OriW1 (Supplementary data Fig. S1). Consequently, anthocyanin derivatives were produced only in the pure white areas where the DvIVS gene was expressed, and all of the cultivars that did not express the DvIVS gene could not produce anthocyanin derivatives. These results indicate that all of the structural genes, except for CHS, are normally expressed in the pure white areas of ‘Yuino’ and OriW2.

The temporal expression of the CHS genes was further characterized. In the red areas of ‘Yuino’, transcripts of DvCHS1 and DvCHS2 were detected regardless of the petal developmental stage, except during the early stage. However, there appeared to be fewer transcripts in the pure white areas than in the red areas (Supplementary data Fig. S2).
Real-time RT-PCR of mature and premature DvCHS1 and DvCHS2 mRNAs

The relative expression levels of the CHS genes were compared among the cultivars by real-time RT-PCR. In ‘Hakuyo’ and ‘Malcoms White’, the DvCHS1 mRNA was not detected but DvCHS2 expression was detected by real-time RT-PCR (data not shown), which was consistent with the RT-PCR results (Fig. 2). In ‘Yuino’, the accumulation of the DvCHS1 and DvCHS2 mRNAs was lower in the pure white areas than in the colored areas by about 1/4 and 1/20, respectively (Figs. 3a, c). Similarly, the accumulation of the DvCHS1 and DvCHS2 mRNAs in the pure white areas of OriW2 was lower by about 1/4 and 1/7, respectively, than in the colored areas. In OriW1, the DvCHS1 mRNA was not detected regardless of petal color, while the DvCHS2 mRNA accumulated in the pure white areas was seven times lower than that in the spontaneously produced yellow areas (Figs. 3a, c).

In the pure white areas of ‘Yuino’ and OriW2, the accumulation of premature mRNA of DvCHS1, considered to reflect the transcription of the DvCHS1 gene was almost the same as or rather higher than that in the colored areas (Figs. 3a, b). In OriW1, premature DvCHS1 mRNA was not detected regardless of petal color. The results were similar for DvCHS2; the accumulation of DvCHS2 mRNA was lower in the pure white areas than in the colored areas, whereas the accumulation of the premature DvCHS2 mRNA was not as low or rather higher in the pure white areas than in the colored areas (Figs. 3c, d).

Analysis of CHS small RNAs

To test whether the PTGS of the CHS genes is mediated by siRNA in the pure white areas of ‘Yuino’, OriW1, and OriW2, we characterized siRNA accumulation in these cultivars using RNA gel blot analysis. Using fragmented antisense DvCHS1 as a probe, siRNA of approximately 24 nucleotides was detected in the pure white areas of ‘Yuino’, OriW1, and OriW2, whereas siRNA was not detected in ‘Hakuyo’ and ‘Malcoms White’ and in the colored areas of ‘Yuino’, OriW1, and OriW2 (Fig. 4).

In our preliminary study, the sequencing of DvCHS1 and DvCHS2 transcripts expressed in ‘Yuino’ bicolor petals was performed to determine the sequence of the redundant allele. We found two sequences for each CHS gene: DvCHS1-1 and DvCHS1-2 for DvCHS1 and DvCHS2-1 and DvCHS2-2 for DvCHS2. Comparing DvCHS1-1 and DvCHS2-1, which share 69% identity with the nucleotide sequence in the open reading frame (ORF), the longest continuous identical sequence consisted of 17 nt (Supplementary data Fig. S3). If all four genes were compared, the longest continuous identical sequence was 14 nt. Phylogenetic analysis of the nucleotide sequences in the ORFs of higher plants’ CHS, including dahlia, petunia, and soybean, showed that DvCHS1 and DvCHS2 belong to a relatively distant subgroup than PhCHSA and PhCHSJ or GmCHS3 and GmCHS7 (Fig. 5).

To ensure that the two phylogenetically different CHS genes are post-transcriptionally silenced by siRNAs, a deep sequencing analysis of the small RNAs accumulating in the pure white areas of ‘Yuino’ was performed. Out of a total of 26,849,283 sequenced small RNAs, about 65% (17,455,041) were 18–32 nt in length and 24 nt was dominant (42% of 18–32 nt). When no mismatch was allowed, small RNAs identical to DvCHS1-1, DvCHS1-2, DvCHS2-1, and DvCHS2-2 were detected (Fig. 6). In addition, in all CHS genes, almost all small RNAs derived from sense and antisense strands were mapped on exon 2, as reported in other species (De Paoli et al. 2009; Kurauchi et al. 2009; Tuteja et al. 2009) (Fig. 6).
Discussion

*DvCHS1* and *DvCHS2* are post-transcriptionally suppressed in the pure white area

As no flavonoid derivatives were detected in the white areas of ‘Yuino’, OriW1 and OriW2 (Table 3), their white areas are pure white in contrast to those of commercial white cultivars. The expressions of *DvCHS1* and *DvCHS2* were low in the pure white areas of ‘Yuino’ and OriW2, and that of *DvCHS2* was low in OriW1, compared to that in the colored areas (Figs. 2, 3a, 3c). We could detect *DvCHS2* transcripts in the commercial white cultivars ‘Hakuyo’ and ‘Malcoms White’, at levels 3.8 and 4.2 times greater than those in the pure white areas of ‘Yuino’, respectively (data not shown). These results suggest that the pure white areas in ‘Yuino’, OriW1 and OriW2, developed due to the reduction in the level of *DvCHS1* and *DvCHS2* mRNAs. We also analyzed the transcription levels of premature *DvCHS1* and *DvCHS2* mRNA in the colored and pure white areas of ‘Yuino’, OriW1 and OriW2, because PTGS usually occurs only in mature mRNA and not in premature unspliced mRNA. Primers for mature mRNAs can also detect premature mRNAs. However, since the amounts of premature mRNAs were assumed to be much lower than those of mature mRNAs, we regarded the score to be similar to that of mature mRNAs. The results revealed that the quantity of premature *DvCHS1* mRNA was nearly the same in the pure white and colored areas of ‘Yuino’ and relatively higher in the pure white areas of OriW2 (Fig. 3c). The difference in *DvCHS2* between the pure white and colored areas was lower for premature mRNA than for mature mRNA in ‘Yuino’ and OriW1, and the amount of premature mRNA in the pure white area was higher than that in the colored areas in OriW2 (Fig. 3d). Suppression of the expression levels (mature mRNAs) of *DvCHS1* and *DvCHS2* was greater than the decrease in the transcription levels (premature mRNAs) in the pure white areas. These results indicate that the transcription of both *DvCHS1* and *DvCHS2* occurs normally in the colored and pure white areas, while the suppression of mRNA occurs post-transcriptionally in the pure white areas. The decrease in premature mRNA levels observed in the pure white areas of ‘Yuino’ and OriW1 may be caused by epigenetic modifications, because endogenous siRNA induces not only target RNA cleavage but also epigenetic modifications, such as DNA methylation and histone modification (Ghildiyal and Zamore 2009).

All *CHS* are silenced by siRNA in the pure white areas

In transgenic plants, multiple *CHS* are suppressed by RNA interference or cosuppression. In petunia, suppression of *PhCHSA* suppressed both *PhCHSA* and *PhCHSJ* (De Paoli et al. 2009). Because the two genes have high sequence similarities, a specific sequence of siRNA is considered to induce simultaneous silencing of both *PhCHSA* and *PhCHSJ*. The homologous transgene-induced PTGS of *CHS* resulting in white flowers is also reported in petunia (Napoli et al. 1990; Van Der Krol et al. 1990). Furthermore, when RNA interference targeted to the 3′ untranslated region of a *CHS* was induced in *Trenia* plants, which have at least two *CHS*, gene-specific silencing was observed and the other *CHS* was not suppressed, whereas when RNA interference was targeted to the relatively conserved *CHS* coding region, simultaneous PTGS was induced (Fukusaki et al. 2004). In case of endogenous *CHS* siRNA, the yellow beans of *G. max* result from PTGS of *CHS*: PTGS was shown to occur in a number of *CHS* by high-throughput sequencing of small RNA (Kurauchi et al. 2009; Tuteja et al. 2009).

The pure white areas of ‘Yuino’, OriW1 and OriW2, accumulated endogenous siRNA carrying
CHS sequences (Fig. 4). siRNA was detected only in the pure white areas. Hence, siRNA-mediated PTGS of CHS is assumed to be the main cause of pure white phenotypic traits. However, RNA gel blot hybridization could not determine whether PTGS occurred in both DvCHS1 and DvCHS2, since the DvCHS1 antisense RNA used as a probe could hybridize to DvCHS1 and DvCHS2 siRNAs. Indeed, siRNA was detectable with the DvCHS1 probe in OriW1, whose transcription of DvCHS1 is inactive. This is believed to be the result of cross-hybridization of the DvCHS1 probe to DvCHS2 siRNA. In fact, it was ascertained that the DvCHS1 probe was able to hybridize to DvCHS2 RNA fragments transcribed in vitro under the same experimental conditions, and that the DvCHS2 probe could also detect DvCHS1 RNA fragments (data not shown).

We isolated DvCHS1-1 and DvCHS1-2 mRNAs for DvCHS1, and DvCHS2-1 and DvCHS2-2 mRNAs for DvCHS2 in ‘Yuino’ petals. The nucleotide sequence identity of ORFs in DvCHS1 (DvCHS1-1; AB576660) and DvCHS2 (DvCHS2-1; AB591825) is 69%, which is much lower than that of the CHS alleles analyzed in the petunia (85%; PhCHS6A and PhCHS7) and soybean (81%; GmCHS4 and GmCHS7) reports, and they are actually located in different subgroups in the phylogenetic tree (Fig. 5). In addition, a continuous homologous sequence longer than 17 bp could not be found between DvCHS1-1 and DvCHS2-1 (Supplementary data Fig. S3), and the untranslated regions between them differed greatly. To confirm that both DvCHS1 and DvCHS2 are post-transcriptionally silenced by siRNAs, a deep sequencing analysis was performed. The small RNAs that accumulated in the pure white areas of ‘Yuino’ were mapped on DvCHS1-1 and DvCHS1-2 or DvCHS2-1 and DvCHS2-2 (Fig. 6). These results strongly indicate that the detected CHS siRNAs (Fig. 4) contained both DvCHS1 and DvCHS2, and that all alleles of DvCHS1 and DvCHS2 were silenced in the pure white area of ‘Yuino’. Almost all small RNAs were mapped on exon 2 of DvCHS1 and DvCHS2 (Fig. 6), as in soybean and petunia (De Paoli et al. 2009; Kurauchi et al. 2009; Tuteja et al. 2009), suggesting that the main cleavage site is located on exon 2. We could not confirm whether the silencing of DvCHS1 and DvCHS2 is interdependent. However, we could detect CHS siRNA from the pure white area of OriW1, which did not express DvCHS1 in the petals, demonstrating that the silencing of DvCHS2 can be induced without DvCHS1 in OriW1. In conclusion, these results indicate that the siRNA-mediated PTGS of two CHS belonging to different subfamilies produces pure white expression in dahlia.

The mechanisms for pure white expression in dahlia

Flavone derivatives were detected in the ivory white petals of the commercial white cultivars. In contrast, no flavonoid derivatives were detected in the pure white areas of star-type cultivars, OriW1 and OriW2. Thus, the difference between ivory white and pure white flower expression is due to biosynthesis and accumulation of flavone derivatives. The accumulation of flavone derivatives in commercial white cultivars is consistent with the results of a previous report that showed that the butein biosynthesis pathway is nonfunctional in white cultivars, and that butein is not a possible causative compound for the ivory phenotype (Nordström and Swain 1958). The major anthocyanidins of dahlia are cyanidin and pelargonidin derivatives (Bate-Smith et al. 1955; Saito et al. 1970) and flavones, such as apigenin and luteolin derivatives, are always observed in these anthocyanic cultivars (Lawrence 1929). In addition to these pigments, chalcone derivatives, such as butein and isoliquiritigenin derivatives, which were reported to be the major chalcone derivatives in yellow cultivars (Nordström and Swain 1956; Saito et al. 1970) were detected in the colored areas of anthocyanic star-type cultivars, except for ‘Kazusa-shiranami’, which did not accumulate chalcones.

In our previous research, we showed that DvIVS regulates DvCHS1, DvF3H, DvDFR, and
DvANS, but not DvCHS2 and DvCHI, and that anthocyanin synthesis and butein synthesis are controlled separately from the anthocyanin synthesis in ‘Michael J’, which displays orange variegation in yellow petals (Ohno et al., unpublished data). We could detect DvIVS expression in anthocyanin-accumulating cultivars but not in non-anthocyanin-accumulating cultivars, such as commercial white cultivars and OriW1, indicating that transcriptional regulation of DvIVS can be generally applied to the garden dahlia. Taking these findings together, we can explain the mechanism for pure white and ivory white flower expressions in dahlia. By both the anthocyanin synthesis and butein synthesis pathways, flavone synthase (presumably DvFNS) was expressed, and petals accumulated anthocyanins, chalcones, and flavones, as in the star-type cultivars (Fig. 7a). When the butein synthesis pathway is suppressed, the petals accumulate anthocyanins and flavones, as in the purple petals of ‘Kazusa-shiranami’. When DvIVS expression is suppressed, petals accumulate chalcones and flavones, as in the OriW1 yellow areas (Fig. 7b). When DvIVS expression and the butein synthesis pathway are suppressed, petals accumulate only flavones, as in the commercial white cultivars (Fig. 7c). When DvCHS1 and DvCHS2 are simultaneously suppressed, no flavonoid derivatives accumulate in the petals, producing pure white areas, such as the white areas of ‘Yuino’ and OriW2 (Fig. 7d). DvCHS2 expression is only detected in the yellow areas of OriW1, due to a combination of CHS siRNA-mediated suppression and non-expression of DvIVS; only DvCHS2 is degraded by siRNA because DvCHS1 is not expressed. In feeding experiments, ‘Yuino’ and OriW2 produced anthocyanin when taxifolin or naringenin was fed to their pure white petals (Supplementary data Fig. S2, data not shown), suggesting that downstream of CHS are functional. This result supports our explanation. Moreover, differences in the expression of DvCHI, DvF3H, and DvDFR between the pure white and colored areas were not observed using real-time RT-PCR in the star-type cultivar ‘Matsuribayashi’ (data not shown). These results indicate that suppression of the CHS contributes to the pure white trait (Fig. 7d).

Pure white flowers of dahlia

Suppression of the structural and regulatory genes for anthocyanin biosynthesis induces white flower coloration in Antirrhinum majus (Martin et al. 1985), I. nil (Morita et al. 2006; Hoshino et al. 2009), D. caryophyllus (Mato et al. 2000), and Gentiana (Nakatsuka et al. 2005). Termination of the anthocyanin biosynthesis pathway at its initial step leads to pure white flower coloration. Thus, it may also be possible to produce a pure white cultivar in dahlia, if all CHS are silenced. However, despite a huge numbers of cultivars being produced, there are no pure white cultivars. We suggest two possible reasons for this, 1) gene redundancy in dahlia and 2) the lack of vigor in pure white cultivars as described below.

In several cases, gene redundancy resulting from polyploidy may have a beneficial effect on the diversification of plants, but this makes it difficult to breed knock-out plants. If there are redundant genes, even if a mutation occurs in a single gene, other genes can compensate for the mutated gene function. Because garden dahlias are autoallooctoploids (Gatt et al. 1998), they may have redundant genes derived from different subfamilies. Therefore, in order to breed pure white dahlia cultivars, all CHS belonging to different subfamilies must be suppressed. Dhalias have at least two CHS subfamilies and several alleles for each gene, making it difficult to accumulate loss-of-function mutations. This is one of the reasons for absence of any pure white cultivars in dahlia. One way to overcome gene redundancy is by inducing PTGS silencing to all the redundant genes. In the yellow bean cultivar of G max, an inverted repeat of CHS in the genome causes endogenous PTGS (Senda et al. 2002). In addition, in a marginal picotee cultivar of petunia, the direct repeat of PhCHSA is assumed to be a trigger of CHS PTGS (Stam, 1997). Thus, genetic mechanisms that exist at PTGS of CHS should also exist in the star-type cultivars,
OriW1 and OriW2. Although we did not elucidate such mechanisms in pure white plants, investigation of the mechanism that induces simultaneous silencing of low homology genes, such as *DvCHS1* and *DvCHS2*, will provide an interesting insight into the breeding of pure white dahlia cultivars, and also to the breeding of polyploidy plants with simultaneous PTGS in several genes with low homology.

Lack of vigor in pure white plants has been reported by Nordström and Swain (1958). They named the pure white plants that had no flavone in their petals as ‘Clare White’; these plants lacked vigor and had a low survival rate. ‘Clare White’ is inferred to have deficiencies in the biosynthesis of flavonoid derivatives. Because flavonoid derivatives have a wide range of biological functions associated with vigorous growth (Hichri et al. 2011), plants that could not synthesize flavonoids have been discarded even though the flowers were pure white in color. Although OriW1 and OriW2 produce pure white flowers, *DvCHS2* is expressed in the leaves, which accumulate flavone derivatives (data not shown). This result indicates that the two *CHS* are specifically suppressed in the flowers of OriW1 and OriW2. Thus, breeding of pure white flower cultivars in dahlia is quite difficult. However, star-type cultivars including OriW1 and OriW2 may be vital in breeding of pure white cultivars.

Reference


Lawrence WJC (1929) The genetics and cytology of Dahlia species. J Genet 21:125-159


### Table 1. Primers used for semi-quantitative RT-PCR

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*K in DvCHI Full-F primer indicates T and G.
Table 2. Primers used to detect mature and premature mRNAs

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+: detected, tr: trace detected, -: not detected

*due to contamination from the cut margin*
Figure legends

Fig. 1 Flowers of star-type cultivars, commercial white cultivars, pure white seedling cultivars, and their seed parents. (a–c) Star-type cultivars a: ‘Yuino’, b: ‘Matsuribayashi’, c: ‘Kazusa-shiranami’; (d–e) Commercial white-flowered cultivars d: ‘Hakuyo’, e: ‘Malcoms White’, f: ‘Orihime’; and (g–j) Pure white varieties and their spontaneously produced colored flowers g: OriW1 pure white inflorescence, h: OriW1 inflorescence with spontaneously produced yellow petals, i: OriW2 pure white inflorescence, and j: OriW2 inflorescence with spontaneously produced red petals. Flowers of commercial white cultivars appeared ivory rather than white. White areas of star-type cultivars appeared pure white. ‘Orihime’ is a labile cultivar which rarely produces red and white petals in an inflorescence. OriW1 and OriW2 spontaneously produce inflorescences with partly or entirely yellow petals and red petals, respectively.

Fig. 2 Semi-quantitative analysis of RNA expression. Numbers above the lanes indicate each cultivar: 1, ‘Hakuyo’; 2, ‘Malcoms White’; 3, white area of ‘Yuino’; 4, red area of ‘Yuino’; 5, white area of OriW1; 6, yellow area of OriW1; 7, white area of OriW2; and 8, red area of OriW2.

Fig. 3 Real-time RT-PCR for mature and premature CHS mRNA expression. a, mature DvCHS1 mRNA; b, premature DvCHS1 mRNA; c, mature DvCHS2 mRNA; and d, premature DvCHS2 mRNA. Bars indicates the mean ± S.E. (biological replications; n = 3). Each bar represents the expression levels relative to ‘Yuino’ and the figures on each bar represent the relative expression levels. In OriW1, mature and premature DvCHS1 mRNA was not detected. DvActin was used as the internal standard and data were calculated as the expression relative to DvActin expression. Standard curves for each gene were prepared from the diluted series of cloned vectors, respectively.

Fig. 4. RNA gel blot hybridization for CHS siRNA detection. 1, ‘Hakuyo’; 2, ‘Malcoms White’; 3, white area of ‘Yuino’; 4, red area of ‘Yuino’; 5, white area of OriW1; 6, yellow area of OriW1; 7, white area of OriW2; and 8, red area of OriW2.

Fig. 5 Neighbor-joining phylogenetic tree of the ORF nucleotide sequences of various CHSs. The bootstrap values of 1,000 retrials are indicated on each branch and the bar indicates a genetic distance of 0.1. The amino acid sequences were acquired from DDBJ database. DvCHS1-1 (AB576660), DvCHS1-2 (AB576661), DvCHS2-1 (AB591825), and DvCHS2-2 (AB591826) in Dahlia variabilis; AtTT4 (NM_121396) in Arabidopsis thaliana; GhCHS1 (Z38096), GhCHS3 (Z38098), and GhCHS4 (AM906210) in Gerbera hybrida; GmCHS3 (FJ770471) and GmCHS7 (AK245977) in Glycine max; InCHSD (AB001818) and InCHSE (AB001819) in Ipomoea nil; and PhCHSA (AF233638) and PhCHSJ (X14597) in Petunia hybrida.

Fig. 6 Mapping of CHS small RNAs. 18–32 nt small RNAs with 100% match to the DvCHS1-1, DvCHS1-2, DvCHS2-1, or DvCHS2-2 genes were mapped on either the sense (pink: above the X-axis) or antisense (blue: below the X-axis) strand. The vertical dotted lines indicate the border between the ORFs and untranslated regions. The total read of 18–32 nt was 17,455,041 reads. The most detected position was 111,237 reads (accounting for 0.64%) at 686 nt in DvCHS2 and 11,006 reads (accounting for 0.063%) at 1051 nt in DvCHS1-1 (identical to 1058 nt in DvCHS1-2).
Fig. 7 Conclusive pathways for ivory white and pure white flower expressions. **a.** Pathway for ‘Yuino’ and OriW2 colored areas. **b.** Pathway for OriW1 colored area. **c.** Pathway for the ivory white flower expression mechanism in commercial cultivars. **d.** Pure white expression mechanisms in white areas of star-type cultivars and OriW2. “X” marks indicate the simultaneous post-transcriptional suppression of DvCHS1 and DvCHS2. Abbreviations are as follows. 3GT, *anthocyanidin 3-glucosyltransferase*; AS, *aureusidin synthase*; ANS, *anthocyanidin synthase*; CH3H, *chalcone 3-hydroxylase*; CHI, *chalcone isomerase*; CHR, *chalcone reductase*; CHS, *chalcone synthase*; DFR, *dihydroflavonol 4-reductase*; F3H, *flavanone 3-hydroxylase*; and FNS, *flavone synthase*. 
Figures

Fig. 1
Fig. 3

(a) DvCHS1 mature mRNA
(b) DvCHS1 premature mRNA
(c) DvCHS2 mature mRNA
(d) DvCHS2 premature mRNA
Fig. 4

1  2  3  4  5  6  7  8

siRNA

rRNA
Fig. 5
Fig. 6

![Graphs showing the number of matched reads for CHS1-1, CHS1-2, CHS2-1, and CHS2-2.](image-url)
Fig. 7
Supplementary datas

**Table S1** The number of mapped CHS small RNAs at SNPs between *DvCHS1-1* and *DvCHS1-2*

**Table S2** The number of mapped CHS small RNAs at SNPs between *DvCHS2-1* and *DvCHS2-2*

**Fig. S1** Semi-quantitative RT-PCR analysis of *DvCHS1* and *DvCHS2* during the stage of petal development

**Fig. S2** Feeding experiment of anthocyanin precursors in white petals

**Fig. S3** Comparison of *DvCHS1* and *DvCHS2* genes

Supplemental figure legends

**Fig. S1** Expression analysis of *DvCHS1* and *DvCHS2* during the stage of petal development. Petals of the commercial white cultivar ‘Hakuyo’ did not express *DvCHS1* at any developmental stage but expressed *DvCHS2* at all stages of development. Clear bands of *DvCHS1* and *DvCHS2* were observed in the red areas in ‘Yuino’. However, in the white areas, the pale bands of both CHS genes were detected. The petals of ‘Yuino’ are not colored at stage 1, so the RNA extraction was conducted using whole petals of stage 1. Stage 1, 0.5 mm of unopened petal; stage 2, two-thirds sized petals of the fully open petals (at the stage of coloration); and stage 3, fully open petals.

**Fig. S2** Feeding experiments of anthocyanin precursors in white areas. Left, just after feeding treatment. Right, one day after treatment. a, ‘Hakuyo’; b, ‘Yuino’; c, OriW1; and d, OriW2. White areas of ‘Yuino’ and OriW2 turned to red in the fed areas, the other cultivars did not express red derivatives (right panels of a, b, c, and d). Red products were identified as anthocyanidin by HPLC. These photographs were those of taxifolin-(flavanonol) fed petals. The results of the naringenin feeding were same as those of taxifolin feeding.

**Fig. S3** Comparison of the two *DvCHS1* and *DvCHS2* genes. The alignment of the nucleotide sequences of two CHS ORFs is shown. Analysis was conducted using ClustalW. The letters in white on a black background indicate the continuum identity sequence of over 10 nucleotides.
Table S1: The number of mapped CHS small RNAs at SNPs between DvCHS1-1 and DvCHS1-2

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- *DvCHS1*
- *DvCHS2*
- *DvActin*
Fig. S2