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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 dahlias (*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 dahlias.

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 genes 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 4ºC. 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. Elutant A was 1.5% phosphate dissolved in water and elutant 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 elutant B at a flow rate of 1 ml min^{-1} at 40ºC. 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 elute 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 (*n*-butanol:acetic acid:water = 4:1:2 v/v/v) and 30% acetic acid (Harborne, 1984).

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 94ºC for 2 min, 30 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 2 min, and a final extension at 72ºC for 10 min. PCR products were cloned into pTAC-1 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 42ºC for 30 min followed by 99ºC 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: 94ºC for 2 min, 30 cycles of 94ºC for 30 s, 55ºC for 30 s, 72ºC for 90 s, and finally 72ºC 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 *DvCHS1* and *DvCHS2*. We revealed two alleles each, *DvCHS1-1* and *DvCHS1-2* for *DvCHS1,* and *DvCHS2-1* and *DvCHS2-2* for *DvCHS2,* for each *CHS* which were expressed in 'Yuino' petals. Consequently, the primers for quantification of *DvCHS1* were designed from the homologous sequences of *DvCHS1-1* and *DvCHS1-2*, and primers for *DvCHS2* were designed from the homologous sequences between *DvCHS2-1* and *DvCHS2-2*. Primers for *chalcone isomerase* (*DvCHI*), *flavanone 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. *Anthocyanidin synthase* (*DvANS*) and *anthocyanidin 3-glucosyltransferase* (*Dv3GT*) (Table 1) were identified in dahlias and primers were selected from our preliminary sequence data. To isolate cDNA clones of *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 *DvCHS1* and *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: 95ºC for 30s followed by 40 cycles of 95ºC for 5 s and 60ºC 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 *DvCHS1* mRNA was designed from a number of genomic sequences of abundantly expressed *DvCHS1* alleles, as previously determined, and the primer set for premature *DvCHS2* mRNA was designed from identical regions of two different genome sequences of *DvCHS2* alleles. The intron lengths were 708bp and 822bp for *DvCHS1* and 96bp and 106bp for *DvCHS2* (Ohno et al. unpublished data). *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 20ºC 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 *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 × 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 Na_2CO_3 and 80 mM $NaHCO_3$; pH 10.2) and incubated at 60ºC 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 42ºC overnight, and the membrane was washed with high- and low- stringency buffers at 42ºC and 25ºC, 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), *ItIVS* 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′-tetrahydroxychalcone) synthesized by CHS spontaneously converts to naringenin in solutions without CHI, we used naringenin instead of chalcone (4,2′,4′,6′-tetrahydroxychalcone). 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, develop 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%; *PhCHSA* and *PhCHSJ*) 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. Dahlias 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.

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Tables

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

	primer		sequence (5'-3')
DvCHS1	Full-F	sense	AATCCCGGTTTCAAGTGATTA
	Full-R	antisense	ATGCAATGTAAGTGACAACATA
DvCHS2	Full-F	sense	TCTTATTACTGCTCGCAATATCTT
	Full-R	antisense	AGTTAGGGCGAAATCGGCATGGTA
FNS	F	sense	CTGCGGGATTTCATATCCG
	R	antisense	GCAAGGCATTGGTTTTGGACT
FLS	F	sense	CCTTCTGCCATTAACTATCAC
	R	antisense	GGTGGATTGTCTTGAGTTATG
DvCHI	Full-F	sense	ATGGCTGGTCTK*GAGGTCGAA
	Full-R	antisense	GGGCTGAGCTTATTTATTAC
DvF3H	Full-F	sense	CATCTTTGAAATGGGACGAGACTTC
	Full-R	antisense	GATTACTCAAAACACTTCATTATTT
DvDFR	Full-F	sense	ATCGGCTCCTGGTTAGTTAT
	Full-R	antisense	CCATTAATAATATGAACTTTATTAA
DvANS	Full-F	sense	TACCTTCCATCCATCATGGTCACTT
	Full-R	antisense	GTTCTTAGAATGACCCAAACAACAA
Dv3GT	Full-F	sense	AAGCAATAAGAAAAATGGCGACTA
	Full-R	antisense	AAGGCTACCACGATGCAACCAACT
DvActin	F80	sense	TGCTTATGTTGGTGATGAAG
	R276	antisense	CCCTGTTAGCCTTAGGATTT
DvMYB1	Full-F	sense	CACAAACATTTTCAATAGAAAATTG
	Full-R	antisense	TAAGAAAGTTATACTCCGTTACATC
DvMYB2	Full-F	sense	ACAAAAAAGATATCAAAAAGAACAA
	Full-R	antisense	AAATCCAAATGAGAATTCATTTCAT
DvR3MYB	Full-F	sense	TTCTCAAGAATTGTATTGAGAAATT
	Full-R	antisense	GATTTGTTAAAAAGACTAATGATAA
DvDEL	Full-F	sense	ATCTCCTCCAATTCATCAAGATTTT
	Full-R	antisense	CCTTTTCTACAGATCAATCAAACAT
DvIVS	Full-F	sense	TTTGACGTAATTTTGGACCTAATTT
	Full-R	antisense	CATCCATTTTTAAATTGTTTGTGGT
DvWDR1	Full-F	sense	TTTCTACTTTTCCAATAATATCACA
	Full-R	antisense	AGATTCTGTAAAACTTAATATGACT
DvWDR2	Full-F	sense	GATACAGATTATCCCCAATTCCAAA
	Full-R	antisense	GTATTCCCAAACCTATATAGGCTAA

*K in *DvCHI* Full-F primer indicates T and G.

Table 2. Primers used to detect mature and premature mRNAs

	primer		purpose	sequence (5'-3')
DvCHS1	mature mRNA	Oligo $dT_{(20)}$	RT	$T_{(20)}$
		F1037	qRT-PCR sense	CATGTGCTAAGCGAATACGG
		R1142	qRT-PCR antisense	CCTCTCCGGTGGTATTGAAC
	premature mRNA	R1142	RT	CCTCTCCGGTGGTATTGAAC
		exon real-time F	qRT-PCR sense	GTGTGCTCCAAAGCGAGTATC
		intron real-time R	qRT-PCR antisense	TGGAAGGTAAAGTTTTCCCAAA
DvCHS2	mature mRNA	Oligo $dT_{(20)}$	RT	$T_{(20)}$
		F669	qRT-PCR sense	GTTTCGTGGACCGACGG
		R762	qRT-PCR antisense	CGCTCAGTTGTCAAGTCC
	premature mRNA	Full-R	RT	AGTTAGGGCGAAATCGGCATGGTA
		exon 2-F	qRT-PCR sense	TCATCGAATATTTAAAATTAGGGT
		intron 2-R	qRT-PCR antisense	CGGAGCCATGTACTCACAGA
DvActin	mature mRNA	Oligo $dT_{(20)}$	RT	$T_{(20)}$
		F80	qRT-PCR sense	TGCTTATGTTGGTGATGAAG
		R276	qRT-PCR antisense	CCCTGTTAGCCTTAGGATTT

Table 3. Flavonoid compositions of petals used in the experiments

Cultivars	Chalcone derivatives		Aurone derivative	Flavanone derivatives	Flavone derivatives		Anthocyanidin derivatives	
	Butein	Isoliquiritigenin	Sulfuretin	Naringenin	Apigenin	Luteolin	Cyanidin	Pelargonidin
'Hakuyo'	-	-	-	-	+	+	-	-
'Malcoms white'	-	-	-	tr	+	+	-	-
'Yuino' white area	-	-	-	-	-	-	tr[z]	tr[z]
'Yuino' red area	+	tr	tr	-	+	+	+	+
'Matsuribayashi' white area	-	-	-	-	-	-	tr[z]	tr[z]
'Matsuribayashi' red area	+	tr	tr	-	+	+	+	+
'Kazusa-shiranami' white area	-	-	-	-	tr[z]	tr[z]	tr[z]	tr[z]
'Kazusa-shiranami' red area	-	-	-	-	+	+	+	+
OriW1 white petal	-	-	-	-	-	-	-	-
OriW1 yellow petal	+	tr	+	-	+	+	-	-
OriW2 white petal	-	-	-	-	-	-	-	-
OriW2 red petal	+	tr	tr	-	+	+	+	+

+: detected tr: trace detected -: not detected

[z]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. OriW1 and OriW2 were selected from several seedlings of 'Orihime' as the seed parent, the flowers of which 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

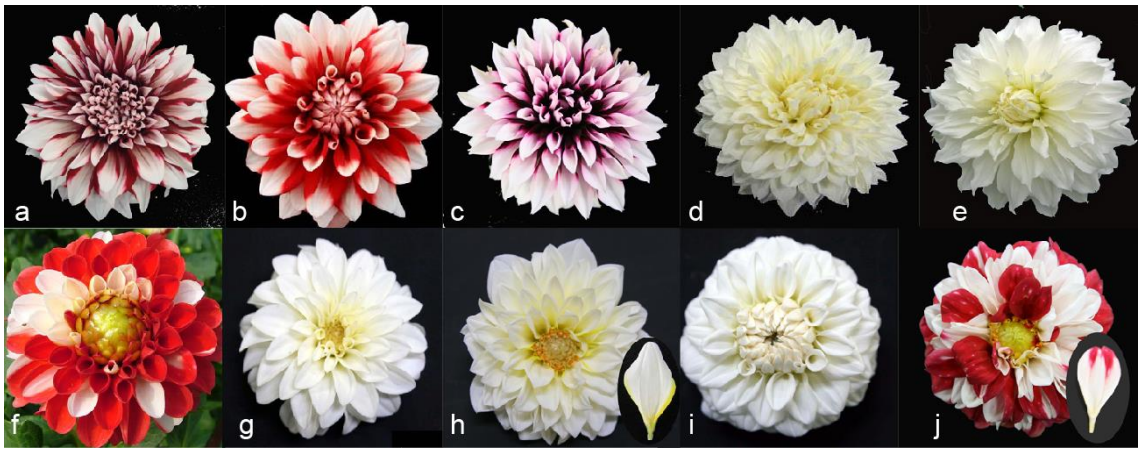


Fig. 2

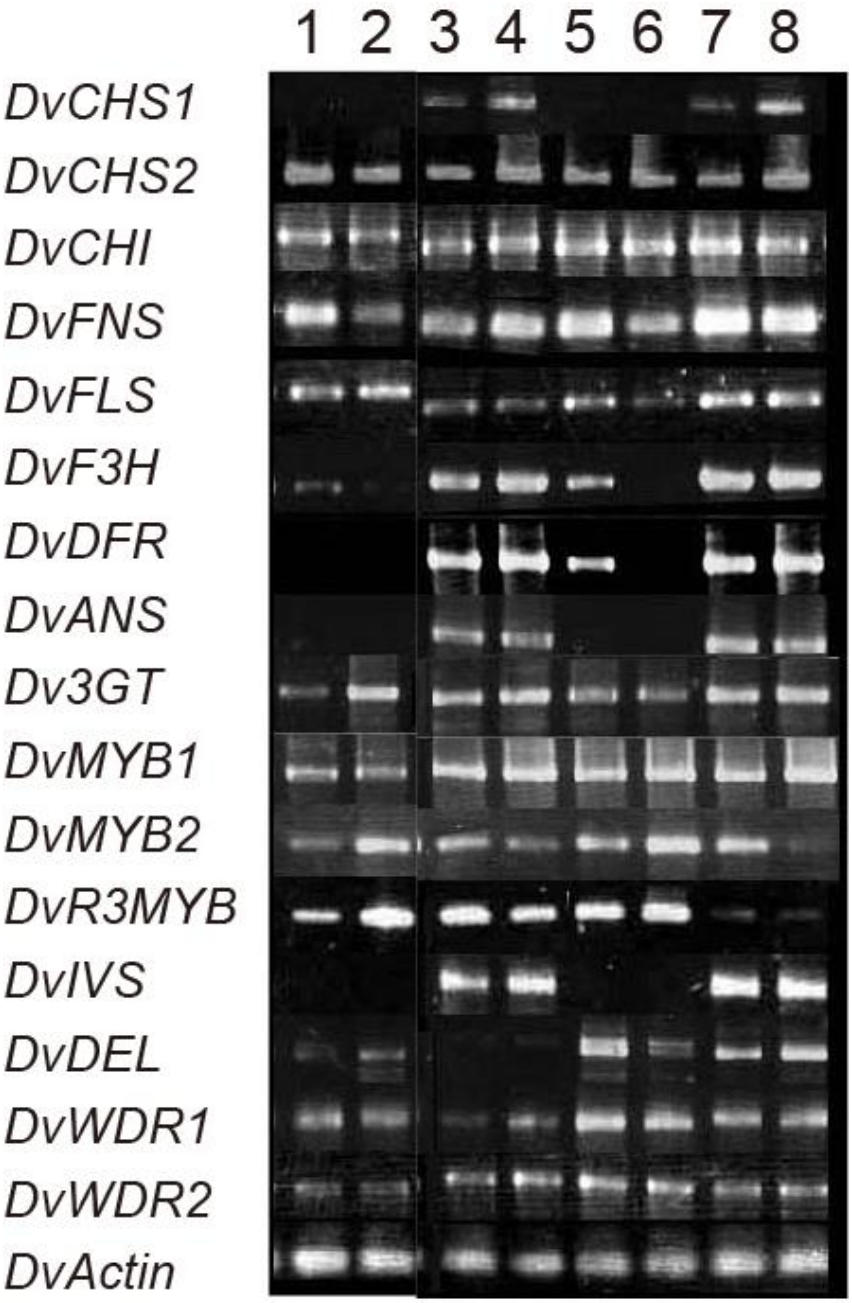

1 2 3 4 5 6 7 8
DvCHS1
DvCHS2
DvCHI
DvFNS
DvFLS
DvF3H
DvDFR
DvANS
Dv3GT
DvMYB1
DvMYB2
DvR3MYB
DvIVS
DvDEL
DvWDR1
DvWDR2
DvActin

Fig. 3

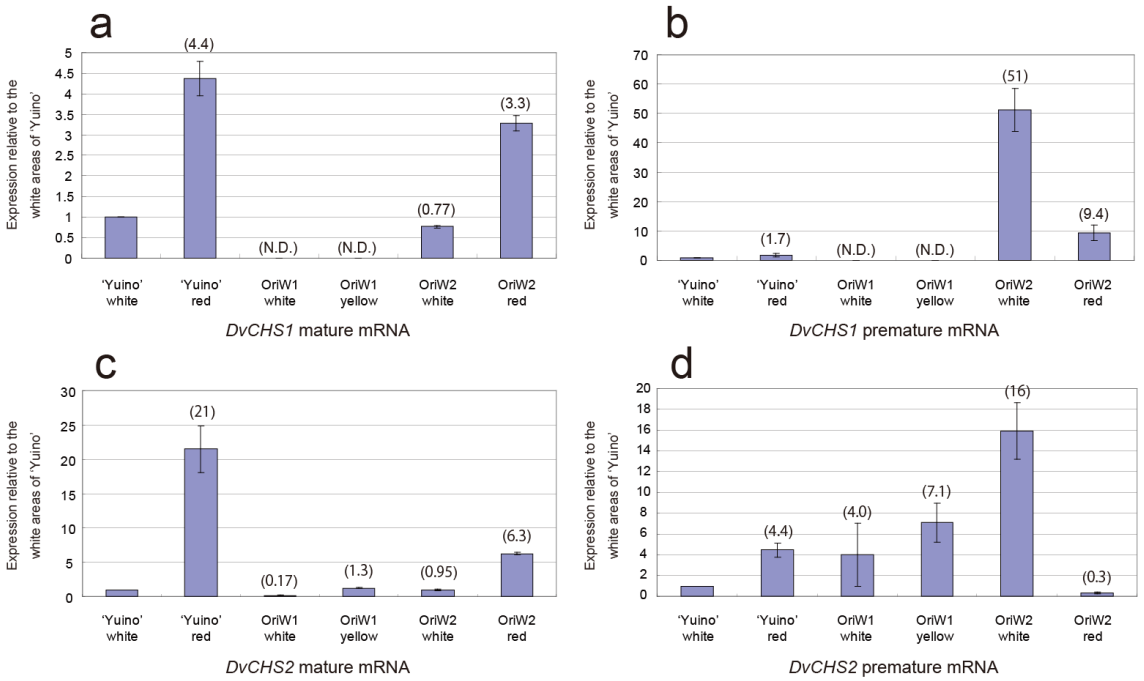

a
Expression relative to the white areas of 'Yuino'
(4.4)
(N.D.)
(N.D.)
(0.77)
(3.3)
'Yuino' white
'Yuino' red
OriW1 white
OriW1 yellow
OriW2 white
OriW2 red
DvCHS1 mature mRNA
b
(1.7)
(51)
(9.4)
DvCHS1 premature mRNA
c
(21)
(0.17)
(1.3)
(0.95)
(6.3)
DvCHS2 mature mRNA
d
(4.4)
(4.0)
(7.1)
(16)
(0.3)
DvCHS2 premature mRNA

Fig. 4

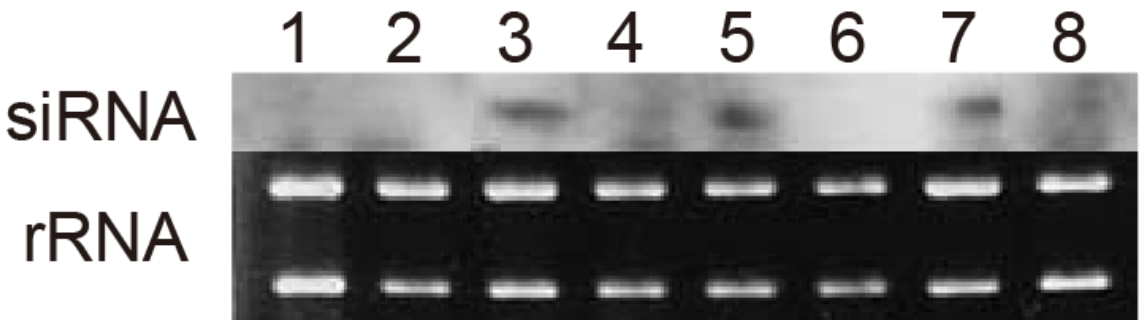


Fig. 5

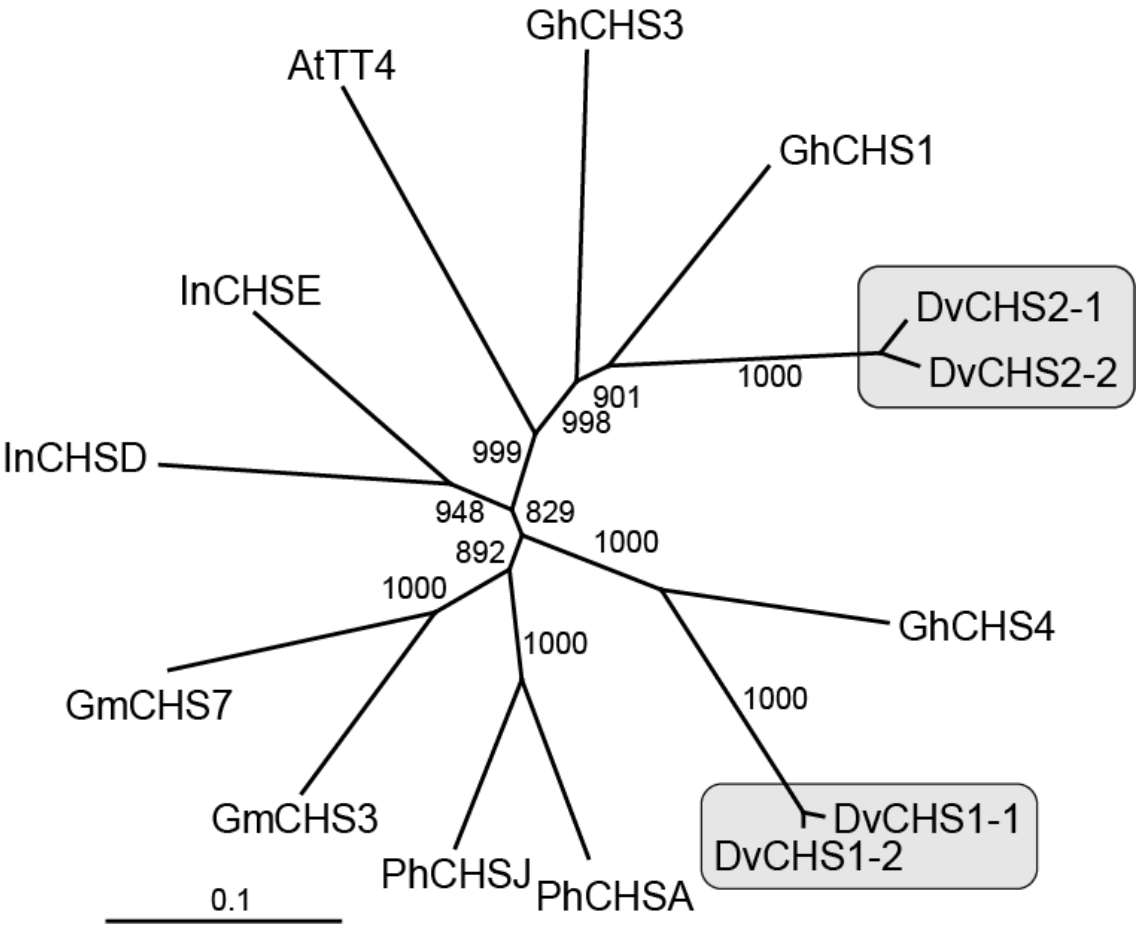

GhCHS3
AtTT4
GhCHS1
InCHSE
DvCHS2-1
DvCHS2-2
1000
901
998
InCHSD
999
948
829
892
1000
1000
GhCHS4
1000
1000
GmCHS7
GmCHS3
DvCHS1-1
DvCHS1-2
PhCHSJ
PhCHSA
0.1

Fig. 6

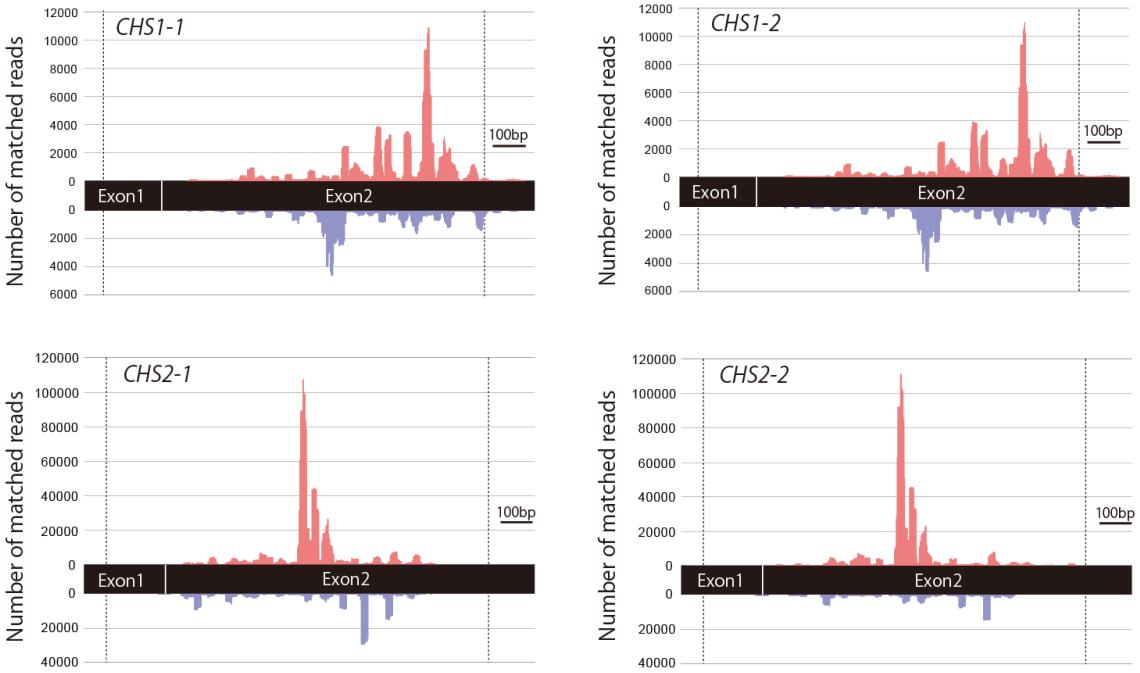
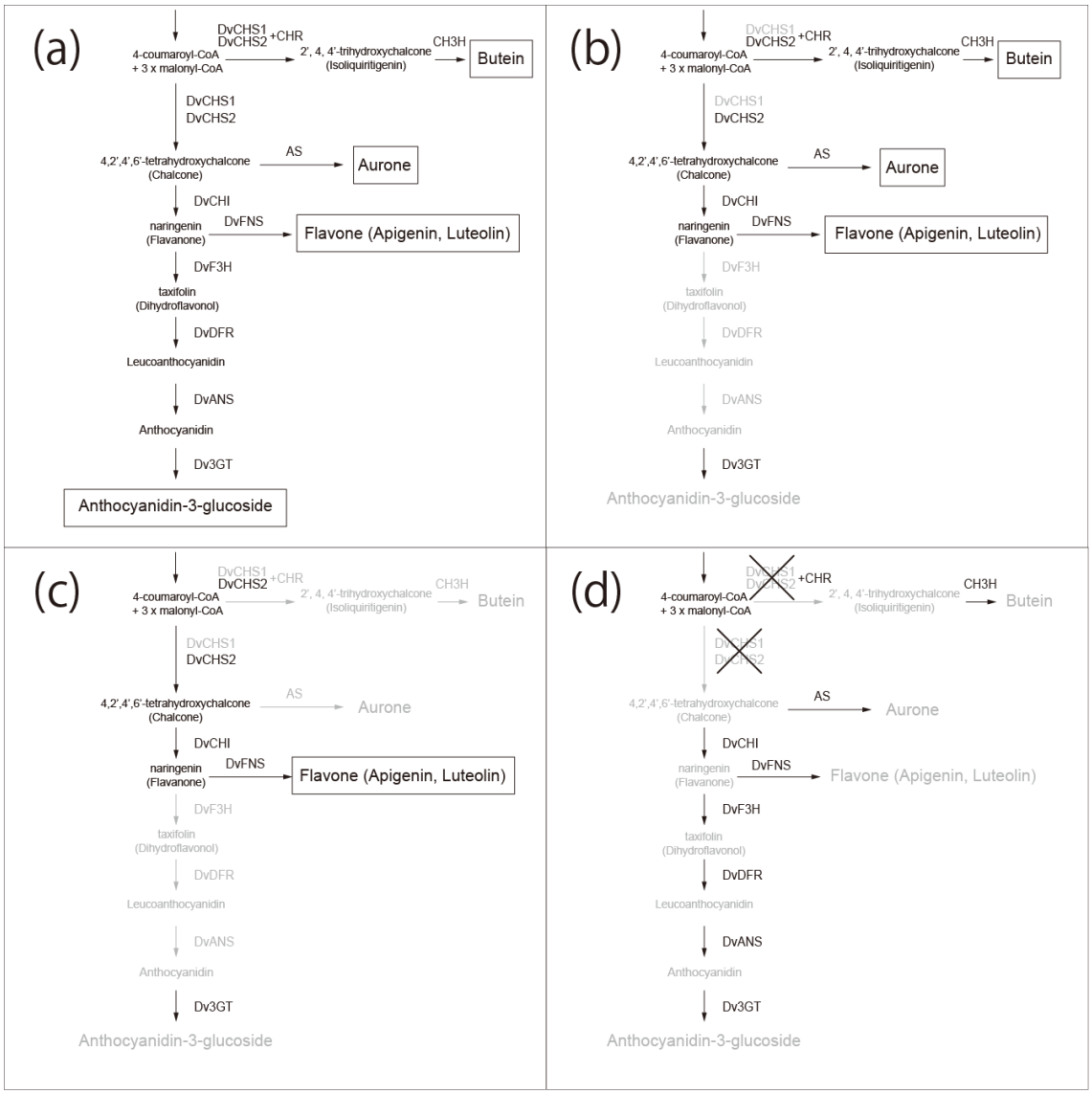

CHS1-1
CHS1-2
CHS2-1
CHS2-2
Number of matched reads
Exon1
Exon2
100bp

Fig. 7


(a)
4-coumaroyl-CoA
+ 3 x malonyl-CoA
DvCHS1
DvCHS2
+CHR
2', 4, 4'-trihydroxychalcone
(Isoliquiritigenin)
CH3H
Butein
DvCHS1
DvCHS2
4,2',4',6'-tetrahydroxychalcone
(Chalcone)
AS
Aurone
DvCHI
naringenin
(Flavanone)
DvFNS
Flavone (Apigenin, Luteolin)
DvF3H
taxifolin
(Dihydroflavonol)
DvDFR
Leucoanthocyanidin
DvANS
Anthocyanidin
Dv3GT
Anthocyanidin-3-glucoside
(b)
(c)
(d)

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.

Supplementary datas

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

DvCHS1-1	37G	-	124C	196G	202T	258C	418T	580A	601G	610C	613T	622G	656A	772C	955A	994G	1023A	1153G	1183G	1236T	1272C
DvCHS1-2	37A	40-46TCTAAAA	131T	203A	209C	265T	425G	587G	608C	617A	620C	629C	663G	779T	962G	1001A	1030G	1160C	1190A	1243C	1279T
1-1 sense	0	0	0	16	12	0	52	335	49	46	447	492	88	387	232	3321	680	30	1036	89	31
1-2 sense	0	0	7	8	8	15	38	314	24	130	232	319	58	386	141	1026	1394	59	1813	73	36
1-1 anti	0	0	0	0	0	0	88	71	32	44	46	127	393	898	92	136	701	62	69	38	43
1-2 anti	1	1	1	3	2	4	82	40	29	60	75	233	104	813	85	161	156	47	194	29	110

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

DvCHS2-1	151T	160G	298T	322G	325T	328C	331T	349T	406G	409T	412C	436A	484T	523T	526T	529C	598T	625G	628A	640T
DvCHS2-2	151C	160C	298C	322C	325C	328T	331C	349C	406T	409G	412G	436G	484C	523A	526G	529T	598C	625A	628C	640C
2-1 sense	8	13	200	1164	1269	1260	862	622	4523	3416	3361	154	1826	1190	1161	2295	1040	2568	2509	401
2-2 sense	1	0	327	43	59	62	53	54	144	113	98	34	2179	175	165	2793	316	3228	3219	183
2-1 anti	0	6	157	3426	2273	2186	2073	9746	1630	308	258	626	387	2310	2704	2739	860	673	717	869
2-2 anti	0	2	126	178	120	108	138	512	136	113	111	1534	226	517	276	268	218	214	120	219

DvCHS2-1	658T	736G	770A	811G	829G	868G	880T	889T	919G	952G	1003G	1024C	1042A	1052A	1075T	1141A	1148G	1222G	1237T
DvCHS2-2	658C	736T	770G	811A	829C	868T	880C	889C	919A	952A	1003A	1024T	1042G	1052C	1075C	1141G	1148T	1222T	1237C
2-1 sense	1611	2436	11792	1440	1535	463	776	662	4708	1448	598	1639	5761	1560	750	118	67	148	2
2-2 sense	639	873	7114	1	958	688	523	546	1303	383	178	893	2052	970	1653	246	244	1049	1
2-1 anti	850	319	2413	9165	676	30817	29346	193	656	15674	361	3441	927	625	178	50	46	15	38
2-2 anti	260	1663	1267	2	286	7696	6890	103	280	14668	669	1746	169	182	100	20	17	1	14

Fig. S1

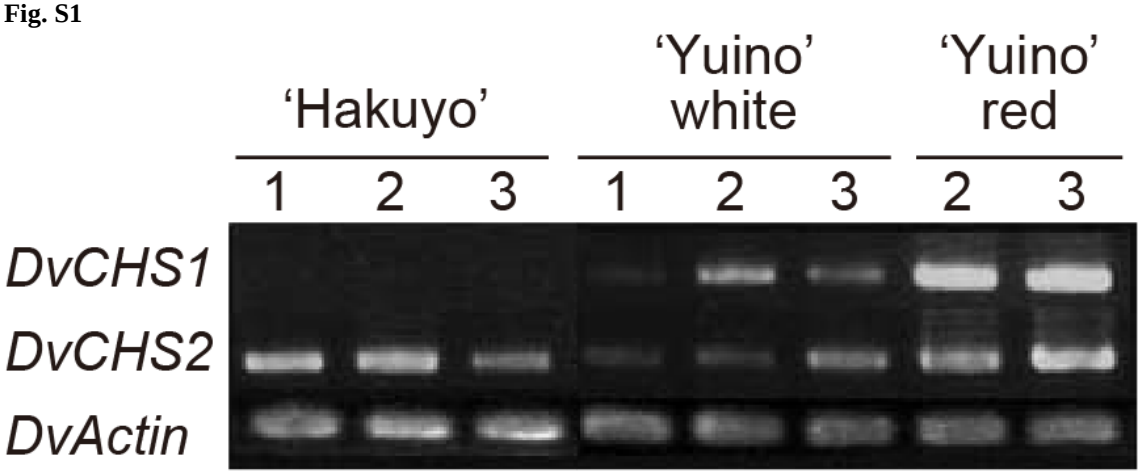

'Hakuyo'
'Yuino' white
'Yuino' red
1 2 3 1 2 3 2 3
DvCHS1
DvCHS2
DvActin

Fig. S2

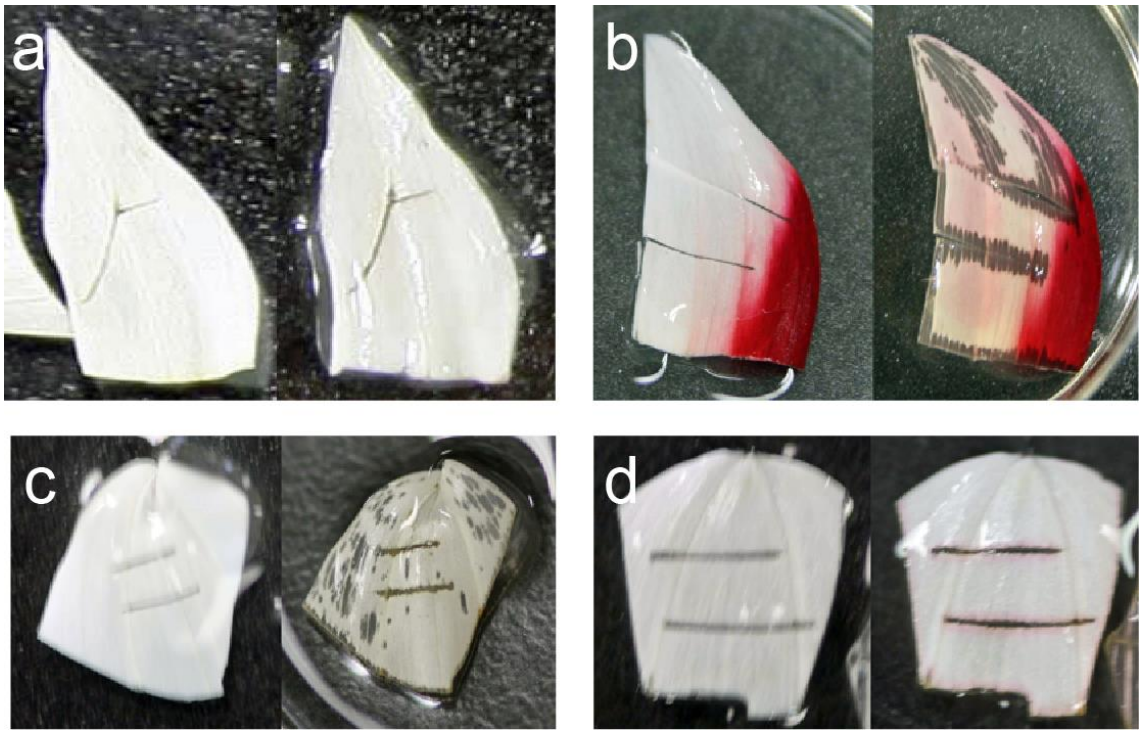


Fig. S3