

White Marginal Picotee Formation in the Petals of *Camellia japonica* ‘Tamanoura’

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Pigment and molecular analyses were carried out to elucidate how the white marginal picotee in the petals of *Camellia japonica* ‘Tamanoura’ is expressed. HPLC analyses showed that two major anthocyanins (cyanidin 3-glucoside and cyanidin 3-galactoside) were accumulated in the red part of the petals of ‘Tamanoura’, as found in those of wild type *C. japonica*, whereas no anthocyanins were detected in the white picotee part, indicating that the anthocyanin biosynthetic pathway might be blocked at some steps in the white part. Transcriptional levels of the genes involved in anthocyanin biosynthesis were investigated by RT-PCR. Most genes were equally expressed in both red and white parts of ‘Tamanoura’ petals, but the expression of *chalcone synthase* (*CHS*) was strongly suppressed only in the white picotee part. Full-length cDNA sequence of *CjCHS* was determined using the 5' and 3' RACE approach. The deduced amino acid sequence of *CjCHS* shared high homology with those of several woody plants. cDNA RT-PCR and genomic DNA PCR revealed no length differences in PCR products between red and white picotee parts in the petals of ‘Tamanoura’, suggesting that no insertion of transposable elements and DNA rearrangement occurred in *CjCHS* in the white part.

Key Words: anthocyanin, *Camellia japonica*, chalcone synthase, marginal picotee, RT-PCR.

Introduction

Flower color is caused by major pigments, namely, carotenoids, flavonoids, and betalains. The flavonoids represent a large class of secondary plant metabolites and are phenylpropanoid compounds of great variation in structure and function (Davies, 2009; Holton and Cornish, 1995). Anthocyanins, the most conspicuous class of flavonoids, provide the basis for nearly all pink, red, orange, scarlet, purple, and blue–black flower colors.

The multicolored flowering pattern is one of the most important characters in ornamental plants. It is formed by the maldistribution of pigments and such a distinct coloration pattern implies a well-controlled regulation system (Glover, 2007).

Flower coloration patterns can be classified into four major groups: sector, fleck (spot), mottle (marble), and

margin (picotee), of which the formations of sectorial and flecked flowers are generally responsible for the effect of transposable elements (Iida et al., 2004). Colored (or uncolored) sectors expand along the proximal–distal axis according to the direction of cell division in these cases. The difference between them is due to the frequency and timing of the insertion or excision of transposable elements. Mottled flowers have been considered to be involved in viral infection (Brierley and Smith, 1944; McWhorter, 1938).

It is difficult to explain the mechanism of the formation of marginal flowers, which shows a perpendicular pigmented (or unpigmented) tip to the axis. An outstanding classical genetic study of morning glory (*Ipomoea nil*) described that at least three genes were thought to be involved in the formation of picotee flowers (Imai, 1927). Saito et al. (2006) recently reported that the expression of *chalcone synthase* (*CHS*), which catalyses the conversion of cinnamic acid derivatives into flavonoids, was repressed only in the white margins, and concluded that the repression of *CHS* was involved in the formation of white marginal picotee in the corolla of *Petunia*. These studies on marginal flowers are,

Received; September 1, 2009. Accepted; October 21, 2009.

This work was financially supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists and the Otomo Fund of the International Camellia Society (ICS).

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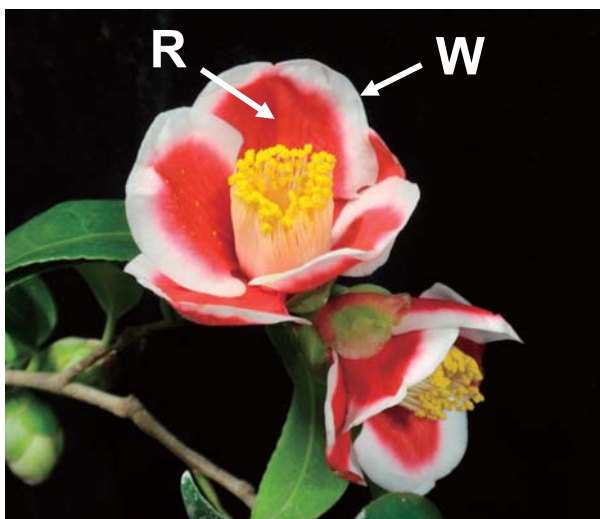


Fig. 1. Flowers of *Camellia japonica* 'Tamanoura'. R, red part in 'Tamanoura' petals; W, white picotee part in 'Tamanoura' petals.

however, limited to herbaceous plants and few cases are known in woody ornamental plants.

Camellia japonica is an evergreen, woody broadleaf tree distributed widely throughout Japan and it has been utilized as ornamental, oil and fuel, plants (Tuyama, 1968). In particular, vast numbers of cultivars have been selected and established for ornamental purposes; consequently, genetic variations in the shapes and colors of flowers and leaves have been accumulated since the Edo era (1603–1867). The breeding program of camellia plants still depends on the selection of seedlings that appear by chance, in contrast to its long cultivation history. Hence, the accumulation of molecular information controlling the flower characters of camellia would contribute to the efficient breeding of flower color, because little is known about the molecular mechanism of flower pigmentation in this plant.

'Tamanoura' is one of the most famous camellia cultivars in the world for its beautiful white marginal picotee on red petals (Fig. 1). It was discovered by chance in a forest in Tamanoura Town, Goto Islands, Nagasaki Prefecture, Japan (Savige, 1993). Elucidating the control system of 'Tamanoura' flower coloration will be helpful to understand the mechanism of picotee flower formation in all plant species, including both herbaceous and woody plants. The aims of this study were to obtain molecular information on camellia flower coloration and to clarify the cause of white picotee formation in the petals of 'Tamanoura' using HPLC and RT-PCR analyses.

Materials and Methods

Plant materials

Wild *C. japonica* accession (collected in Kurose, Fukue Island, Nagasaki Prefecture, Japan) and 'Tamanoura' grown in a greenhouse at Kyushu University were used. Fully expanded flowers were collected immediately at the beginning of anthesis. The

petals of 'Tamanoura' were separated into red and white marginal parts and used for the following experiments. Parts of fresh petals were used for HPLC analyses. Others were frozen promptly using liquid nitrogen and stored at -80°C until used for DNA and RNA extraction.

HPLC analyses

Petal segments (approx. 1 cm^2) were soaked in 1 mL 50% acetic acid overnight to extract anthocyanins. The solutions were filtered through HLC-DISK 25 (hydrophobic, pore size $0.45\text{ }\mu\text{m}$; Kanto Chemical, Tokyo, Japan), and the filtrates were used for analyses using a C-R6A Chromatopac, SCL-6A system controller, SPD-6AV UV-vis spectrophotometric detector, LC-6A liquid chromatograph and a CTO-10A column oven (Shimadzu Corporation, Kyoto, Japan) with a Cosmosil 5C₁₈ MS II packed column ($250\text{ mm} \times 4.6\text{ mm i.d.}$; Nacalai Tesque, Kyoto, Japan) at 40°C with a flow rate of 1.0 mL min^{-1} , monitored at 530 nm. The solvent system was a linear gradient elution from 20% to 59% solvent B [$\text{H}_3\text{PO}_4\text{-HOAc-CH}_3\text{CN-H}_2\text{O}$ (1.5 : 20 : 25 : 53.5)] in solvent A [$\text{H}_3\text{PO}_4\text{-H}_2\text{O}$ (1.5 : 98.5)] for 24 min.

DNA and RNA extraction

The frozen petal tissues described above were used for DNA and RNA extraction. DNA extraction was performed as previously reported (Tateishi et al., 2007). RNA extraction was carried out following Kiefer et al. (2000) with some modifications. Frozen tissues (100–150 mg) were ground to fine powder with a mortar and pestle using liquid nitrogen and $800\text{ }\mu\text{L}$ pre-warmed (65°C) extraction buffer [100 mM Tris-HCl (pH 8.0), 25 mM EDTA , 2 M NaCl , 2% CTAB (w/v), 2% polyvinylpyrrolidone (w/v), 0.5% spermidine (w/v), and 2% β -mercaptoethanol (v/v)] was added followed by incubation at 65°C for 10 min. One hundred μL of Nucleon PhytoPure DNA extraction resin (GE Healthcare, Buckinghamshire, England) and $400\text{ }\mu\text{L}$ chloroform/isoamylalcohol (24 : 1) were added and the sample tubes were kept on a shaker for 10 min at room temperature. After centrifugation at $9,000 \times g$ for 10 min at 4°C , the aqueous phase was washed with $500\text{ }\mu\text{L}$ chloroform/isoamylalcohol (24 : 1) at least three times. The aqueous phase, to which $500\text{ }\mu\text{L}$ isopropanol was added, was incubated on ice for 1 h, followed by centrifugation at $9,000 \times g$ for 10 min at 4°C . RNA pellets were dissolved in $100\text{ }\mu\text{L}$ diethyl pyrocarbonate (DEPC) treated water with 2 M LiCl and incubated at 4°C overnight. After centrifugation at $9,000 \times g$ for 30 min at 4°C , the RNA pellets were treated with DNase I (Roche Diagnostics, Mannheim, Germany) at 37°C for 20 min. An equal volume of isopropanol was added for RNA precipitation, followed by centrifuged at $9,000 \times g$ for 2 min at 4°C . After washing two times with 70% EtOH, the pellets were dried and dissolved in DEPC treated water. The RNA concentration and purity were evaluated at an absorbance of 260 nm.

Design of degenerate and gene-specific primer pairs

Sequence alignments of six anthocyanin biosynthetic structural genes [*phenylalanine ammonia lyase* (*PAL*), *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavanone 3-hydroxylase* (*F3H*), *dihydroflavonol 4-reductase* (*DFR*), *anthocyanidin synthase* (*ANS*)] (Fig. 2) from some higher plants were obtained using DNASIS-Mac v3.2 (Hitachi Software Engineering, Tokyo, Japan). Degenerate primer pairs were designed covering the highly conserved region for each gene (Table 1). Based on the sequences identified (see results for detailed information), four gene-specific primer pairs (*CjCHS*, *CjF3Ha*, *CjDFR*, and *CjANS*) were designed by referring to the Primer3 primer design program (<http://frodo.wi.mit.edu/primer3/>, December 24, 2009) and *CjCHS-full* was constructed to amplify the full length of the *CjCHS* gene (Table 1). 18S rRNA was surveyed as an internal control using primer pairs reported by Sato et al. (2005).

RT-PCR and genomic DNA PCR

cDNA synthesis and RT-PCR were carried out employing a TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (Takara, Otsu, Japan) following the manufacturer's instructions. We mixed two primers in the ratio of the

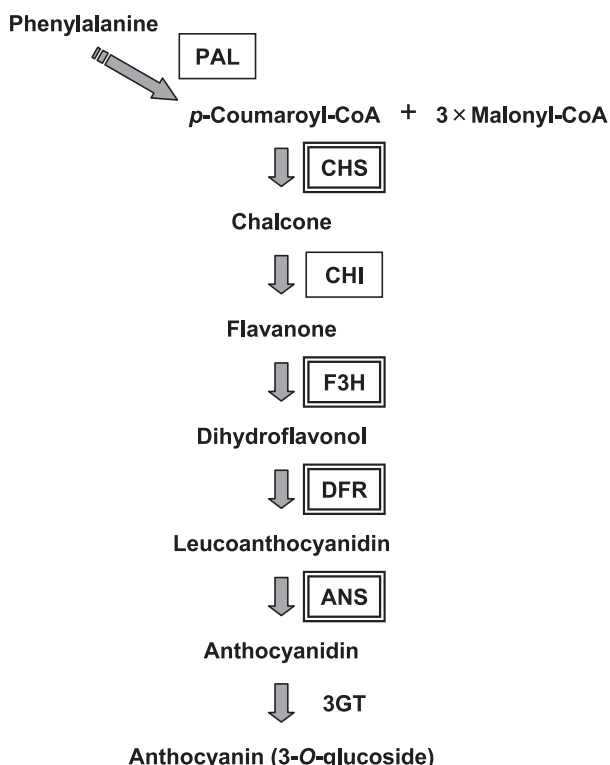


Fig. 2. Outlined scheme of the anthocyanin biosynthetic pathway. The six boxed genes were investigated in Figure 4A by RT-PCR using degenerate primer pairs. The four double-boxed genes were further surveyed in Figure 4B using gene-specific primer pairs. Abbreviations: PAL, phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, flavonoid 3-glucosyltransferase.

Oligo dT-adaptor primer : Random 9 mers = 4 : 1 for reverse transcriptional reaction. PCR amplification was performed in a total volume of 50 μ L containing 45 ng template cDNA, 0.2 μ M of each primer, 10 μ L 5 \times PCR Buffer, 0.2 mM of each dNTP, and 1.25 Unit TaKaRa Ex Taq HS polymerase. For degenerate RT-PCR, the volumes of primer pairs were increased accordingly. Finally, RNase Free dH₂O was added up to 50 μ L. Amplification was conducted using TaKaRa PCR Thermal Cycler Dice TP-600 (Takara) with one cycle of 3 min at 94°C, followed by 27 or 30 or 33 cycles of 20 s at 94°C, 20 s at 56°C, and 1 min at 72°C, and finally one cycle of 10 min at 72°C. PCR products were separated by electrophoresis in 1.5% (w/v) agarose gels and visualized under UV illumination after staining with ethidium bromide. PCR amplification with genomic DNA was conducted in a similar manner with the exception of the PCR cycle, which was as follows: one cycle of 3 min at 94°C, followed by 35 cycles of 20 s at 94°C, 20 s at 56°C, and 2 min at 72°C, and finally one cycle of 10 min at 72°C.

Molecular cloning of *CHS*, *F3H*, *DFR*, and *ANS* in *C. japonica*

Degenerated RT-PCR products of *CHS*, *F3H*, *DFR*, and *ANS* were subcloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into Competent high *Escherichia coli* DH5 α (Toyobo, Osaka, Japan). After culture on LB plates containing 100 μ g·mL⁻¹ ampicillin, 100 μ g·mL⁻¹ X-Gal, and 23.83 μ g·mL⁻¹ IPTG, only white colonies were selected, and then plasmids containing inserts were extracted using a LaboPass Plasmid Mini Purification Kit (Hokkaido System Science, Sapporo, Japan). The BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) and ABI PRISM 310 genetic analyzer (Applied Biosystems) were employed for sequence analyses. To obtain full-length cDNA sequence of *CjCHS*, 5' and 3' rapid amplification of cDNA ends (RACE) was performed using the GeneRacer Kit and TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Cloning and sequence methods were conducted in a similar manner. A phylogenetic tree based on the deduced amino acid sequence of *CjCHS* was drawn using PHYLIP version 3.6 (Felsenstein, 2004).

Results

HPLC analyses of anthocyanins

Two major peaks were detected in the extract from petals of wild *C. japonica* (Fig. 3A), and were identified as cyanidin 3-galactoside (Cy3Ga) and cyanidin 3-glucoside (Cy3G), according to a previous report (Sakata et al., 1986). Two peaks were obviously recognized in the red part of 'Tamanoura' petals, whereas no anthocyanins were observed in the white marginal picotee part (Fig. 3B, 3C). These results clearly indicated

that the anthocyanin biosynthetic pathway is blocked at some step in the white picotee part of 'Tamanoura' petals.

Expression profiles of anthocyanin biosynthetic genes in the petals of 'Tamanoura'

The expression levels of six genes involved in the anthocyanin biosynthetic pathway (Fig. 2) were investigated primarily using RT-PCR with degenerate primer pairs to address which step of the pathway is suppressed in the white picotee part. The degenerate primer pairs

designed in this study (Table 1) were able to amplify PCR products successfully to the expected sizes. RT-PCR was conducted with at least two cycles (27 and 30 cycles for *PAL*, *CHS*, *CHI*, *F3H*, *DFR*, and 18S rRNA; 30 and 33 cycles for *ANS*) to avoid the plateau effect. Most genes tested showed no differences on transcriptional levels in the red and white picotee parts of 'Tamanoura' petals, but only the transcription of *CHS* in the white picotee part was significantly lower than that of the red part and the petals of wild *C. japonica*

Table 1. Primer pairs designed and used in this study.

Primer	Gene	Sequence	PCR product size (bp)
Degenerate primer	<i>PAL</i>	Forward 5'-GAG TTG CAR CCH AAR GAA GG-3'	725
		Reverse 5'-ACA TCT TGG TTR TGY TGY TC-3'	
	<i>CHS</i>	Forward 5'-GAC TAY CAG MTC ACY AAR CT-3'	611
		Reverse 5'-AAC AAC ACG CAH GCA CTD GAC-3'	
	<i>CHI</i>	Forward 5'-GGT SMN TTT GAG AAG TTC A-3'	347
		Reverse 5'-TTT GCW KCW GGG GAA ACW CC-3'	
	<i>F3H</i>	Forward 5'-ATG TCC GGT GGB AAR AAR GG-3'	674
		Reverse 5'-TTG CTC ATC TTC YTC YKG TAC-3'	
	<i>DFR</i>	Forward 5'-RAG GAY CCY GAG AAT GAR G-3'	645
		Reverse 5'-GCT GTA YTT GAA YTY GAA YCC-3'	
	<i>ANS</i>	Forward 5'-TTG AGT GGS AGG ATT AYT TYT TYC-3'	511
		Reverse 5'-GGT TCG CAG AAV ATH GCC C-3'	
Gene-specific primer	<i>CjCHS-full</i>	Forward 5'-ATT GTC CGG CCA AAA TGG TC-3'	1,162
		Reverse 5'-ACC ACA GTC TCA ACA GTG AG-3'	
	<i>CjCHS</i>	Forward 5'-ACC CAC TTG GTC TTT TGC AC-3'	422
		Reverse 5'-AAT GTA AGG CCC ACT TCA CG-3'	
	<i>CjF3Ha</i>	Forward 5'-ACG GAG ACC TAC AGC GAG AA-3'	357
		Reverse 5'-ATG ATC CGC ATT CTT GAA CC-3'	
	<i>CjDFR</i>	Forward 5'-AAC AAC CCA TTT TCG ACG AG-3'	412
		Reverse 5'-TTG TAC TCG GGC CAT TTC TC-3'	
	<i>CjANS</i>	Forward 5'-ACG CAA AGC AAC TAC GAG G-3'	312
		Reverse 5'-CTA CCG TGT CAC CAA TGT GC-3'	
	18S rRNA ^z	Forward 5'-CAT TCG TAT TTC ATA GTC AGA GGT GAA ATT C-3'	434
		Reverse 5'-TTA ACC AGA CAA ATC GCT CCA CCA ACT AAG-3'	

W = A + T; R = A + G; Y = C + T; K = G + T; M = A + C; S = C + G; B = C + G + T; D = A + G + T; H = A + C + T; V = A + C + G; N = A + C + G + T

^z Sato et al. (2005)

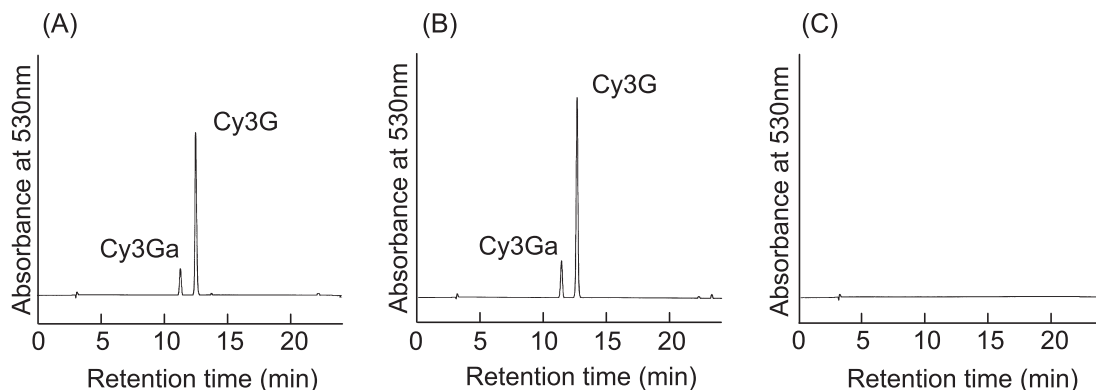


Fig. 3. HPLC chromatograms of anthocyanins in the petals of wild type *C. japonica* and 'Tamanoura'. (A) Petals of wild type *C. japonica*. (B) Red part in 'Tamanoura' petals. (C) White marginal picotee part in 'Tamanoura' petals. Cy3G and Cy3Ga represent cyanidin 3-glucoside and cyanidin 3-galactoside, respectively. Three independent flowers were analyzed per part.

(Fig. 4A). We isolated partial cDNA sequences of *F3H*, *DFR*, *ANS* (GenBank accession nos., *CjF3Ha*, AB524883; *CjF3Hb*, AB524884; *CjDFR*, AB524885; *CjANS*, AB524886) (Tateishi et al., 2009) and the full-length cDNA sequence of *CHS* (next section), and designed gene-specific primer pairs for each gene (Table 1). Gene-specific RT-PCR confirmed the accurate suppression of *CjCHS* in the white picotee part, indicating that this suppression of *CjCHS* is responsible for the formation of the white marginal picotee (Fig. 4B).

Molecular cloning of chalcone synthase in *C. japonica*

Because *CjCHS* showed different expression levels between red and white parts in ‘Tamanoura’ petals, we attempted to obtain its full-length cDNA sequence in *C. japonica*. Using a degenerate RT-PCR strategy and 5' and 3' RACE, we identified one gene, named *CjCHS*,

which encodes a putative CHS protein. The 1,446 bp full-length *CjCHS* cDNA contained an open reading frame (ORF) encoding 390 amino acid residues with a start codon ATG at nucleotide position 84 and a stop codon at position 1,251 (GenBank accession no. AB512766). A phylogenetic tree was constructed to compare the homology of *CjCHS* with related CHSs of other plant species (Fig. 5A). The deduced amino acid sequence of *CjCHS* showed high identity with *Camellia sinensis* (99.7%), and clustered with those of other woody plants. Some important amino acid residues (Cys164 and Phe215) for catalytic activity of CHS, which were strictly conserved in all known CHS-related enzymes (Ferrer et al., 1999), were recognized in the sequence identified in this study (Fig. 5B), supporting that the gene identified here was the homologue of *CHS* in *C. japonica*.

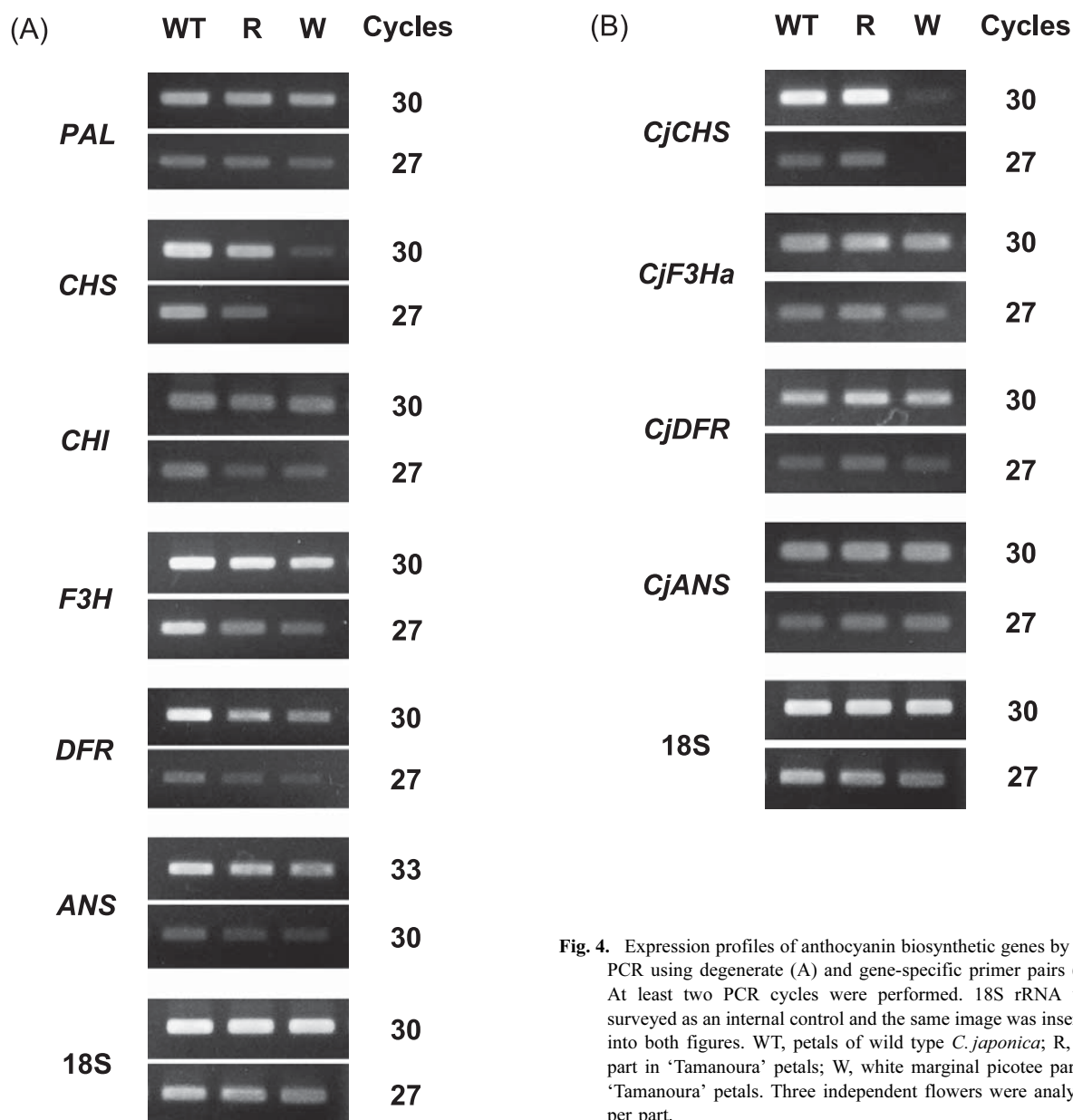


Fig. 4. Expression profiles of anthocyanin biosynthetic genes by RT-PCR using degenerate (A) and gene-specific primer pairs (B). At least two PCR cycles were performed. 18S rRNA was surveyed as an internal control and the same image was inserted into both figures. WT, petals of wild type *C. japonica*; R, red part in ‘Tamanoura’ petals; W, white marginal picotee part in ‘Tamanoura’ petals. Three independent flowers were analyzed per part.

Analyses of the cDNA and genomic DNA structure of *CjCHS*

Based on the sequence of *CjCHS* identified in this study, we designed a *CjCHS*-full specific primer pair by which the whole *CjCHS* gene was amplified (Fig. 6A and Table 1). As expected, approximately 1,170 bp clear bands were observed in the petals of the wild type and the red part of 'Tamanoura' petals with RT-PCR (35 cycles) using cDNA samples (Fig. 6B). An extremely

weak band of the same size was detected in the sample of the white picotee part. PCR amplification with genomic DNA revealed that all three parts have the same size of *CjCHS* gene containing an approximately 300 bp intron (Fig. 6C). No differences in the amplified product size between red and white picotee parts in petals were recognized through cDNA RT-PCR and genomic DNA PCR.

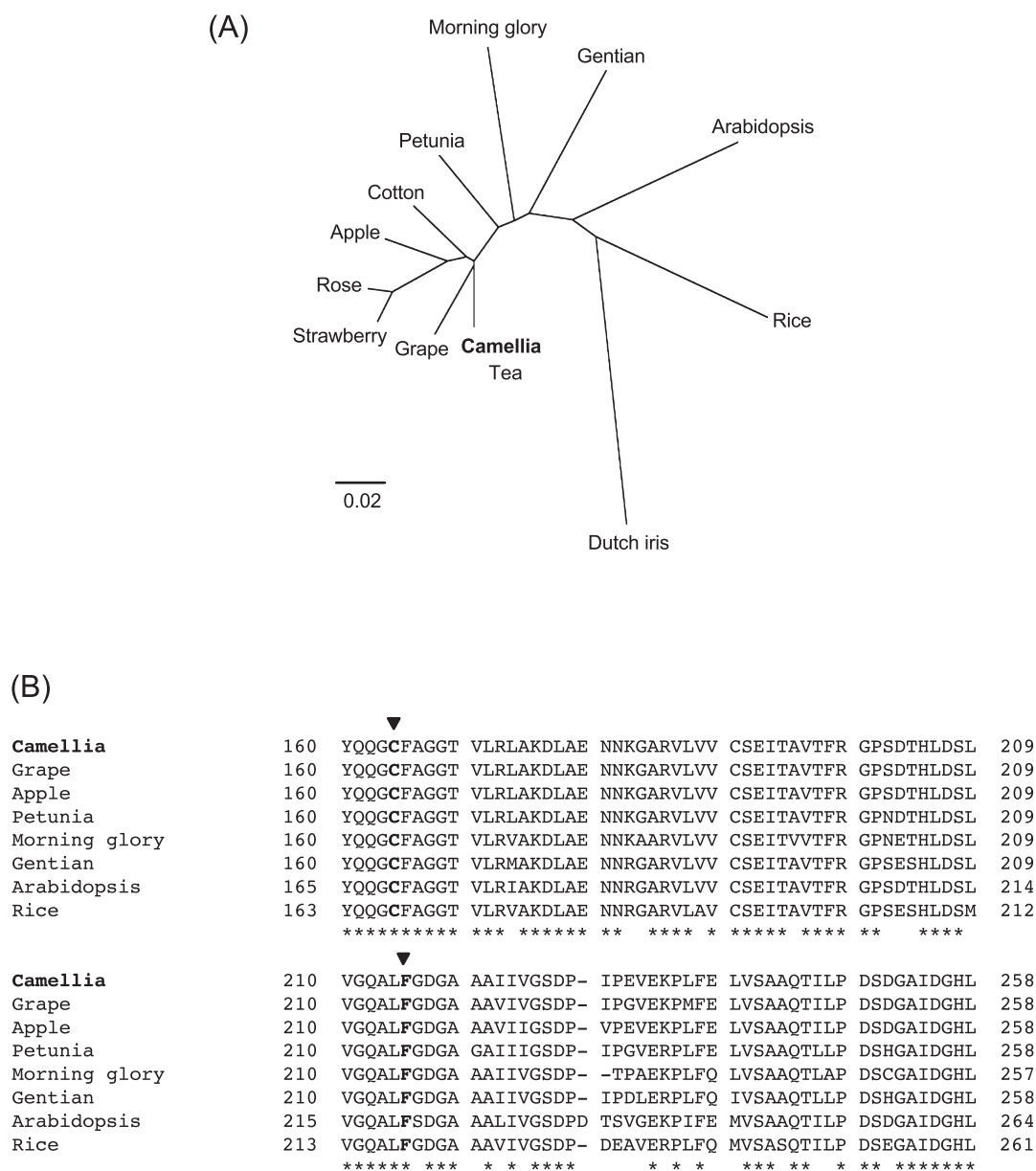


Fig. 5. Comparison of the deduced amino acid sequences of CHSs from higher plants. Plant species names, gene names and their GenBank accession numbers are as follows; arabidopsis [*Arabidopsis thaliana* (MAC12.14), AY044331]; tea [*Camellia sinensis* (CHS1), D26593]; strawberry [*Fragaria × ananassa* (FrCHS2), AB201756]; gentian [*Gentiana triflora*, D38043]; cotton [*Gossypium hirsutum* (CHS), EF643507]; morning glory [*Ipomoea nil* (CHS-D), AB001818]; Dutch iris [*Iris × hollandica* (IhCHS1), AB232914]; apple [*Malus × domestica*, EU872158]; rice [*Oryza sativa*, AB000801]; petunia [*Petunia × hybrida*, X04080]; rose [*Rosa hybrida* (CHS), AB038246]; grape [*Vitis vinifera* (CHS3), AB066274]. (A) A phylogenetic tree for CHS constructed by the neighbor-joining method using full-length deduced amino acid sequences, including camellia [*Camellia japonica* (CjCHS), AB512766]. (B) Comparison of the highly conserved region of CHSs. Asterisk indicates completely identical amino acid residues among eight species. Bold amino acid residues with black arrowheads (Cys164 and Phe215) represent absolute conservation important for catalytic activity.

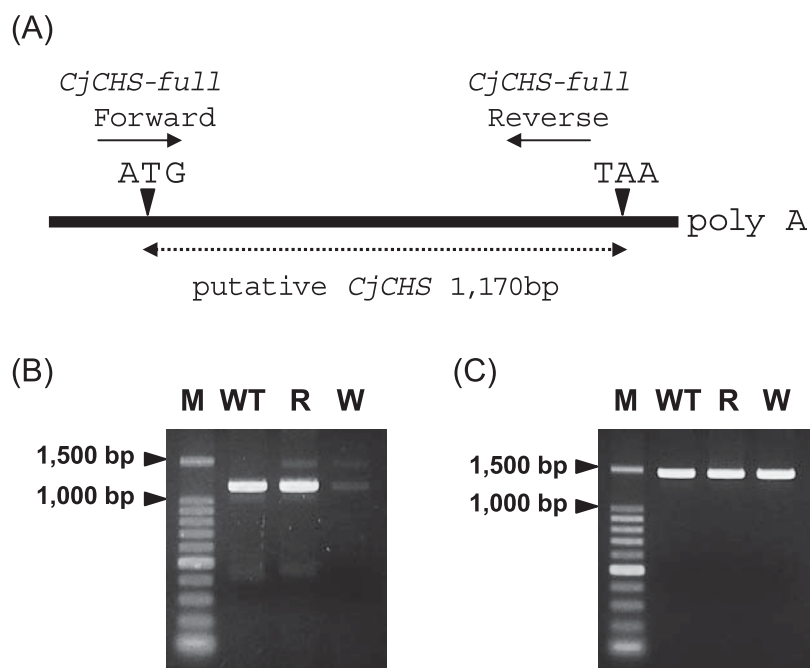


Fig. 6. Analyses of the size of PCR products of *CjCHS* gene. (A) Outlined scheme of the *CjCHS*. *CjCHS*-specific primer pair was designed to amplify the whole length of cDNA and genomic DNA of *CjCHS*. (B) Profiles of RT-PCR products with cDNA samples. Expected size bands were amplified in the petals of wild type *C. japonica* and the red part in ‘Tamanoura’ petals. (C) Profiles of PCR products with genomic DNA samples. Approximately 1,500 bp bands were amplified in all samples. M indicates 100 bp DNA ladder marker. WT, petals of wild type *C. japonica*; R, red part in ‘Tamanoura’ petals; W, white marginal picotee part in ‘Tamanoura’ petals. Three independent flowers were analyzed per part.

Discussion

We demonstrated in this study that the expression level of *CjCHS* was remarkably low in the white picotee part (Fig. 4) and this suppression caused a lack of accumulation of anthocyanins (Fig. 3), resulting in white picotee formation in ‘Tamanoura’ petals. It was also speculated that no insertion of transposable elements and DNA rearrangement occurred in *CjCHS* in the white picotee part (Fig. 6B, 6C). The floral tissues used in this study were restricted to the full blooming stage, because it was difficult to obtain the tiny white picotee part from small buds. Although it is well known that the expression levels of anthocyanin-related genes mainly increase during the bud developmental stage, the obvious suppression of *CjCHS* even at the anthesis stage (Fig. 4) implies the consistent suppression of *CjCHS* throughout the floral bud developmental stage. To our knowledge, this is an unprecedented report on flower color mutation in woody ornamentals. The accumulation of molecular information controlling flower traits described in this study will give valuable insight into the production of new camellias.

Petunia hybrida ‘Red Star’ is a variety whose flowers exhibit white star-type sectors along the vein on the red corolla. The mRNA level of *CHS* is lower in white star sectors than in the red sectors of its corolla (Koes et al., 1989; Mol et al., 1983; van der Krol et al., 1990). Koseki et al. (2005) proved that sequence-specific degradation

induced by post-transcriptional gene silencing (PTGS) is the primary cause of the depression of *CHS* mRNA, resulting in the formation of white star sectors in the corolla of ‘Red Star’.

Saito et al. (2006) investigated the mechanism of white marginal picotee formation in the corolla of *Petunia hybrida* ‘Baccara Rose Picotee’ and reported that the expression of *CHS* was repressed and cinnamic acid derivatives were accumulated in the white margins, while the expression levels of other genes did not change in both parts, suggesting that the reduction in flavonoid biosynthesis by the repression of *CHS* is involved in the formation of an unpigmented zone in the corolla margins. It was recently reported that the formation of white marginal picotee of petunia cultivar is also induced by PTGS of *CHS* (Nakayama, 2009), and the application of a chemical compound released PTGS, resulting in the formation of uniformly colored flowers. (Ban and Nakayama, 2008). In this case, the white marginal picotee part disappeared along the vein tissue, according to the concentration of the chemical compound.

Taken together, both the white parts of the star-type and marginal picotee in petunia corolla seem to be generated from the ‘vein’ tissue. They could be therefore expressed as ‘vein-dependent coloration’. Griesbach et al. (2007) said that the picotee and star-type phenotypes have a similar biochemical basis, and the difference between them is most likely the result of development timing. Namely, the star pattern occurs as a result of

early expression of the mutant gene, whereas the picotee pattern develops by late expression during flower development.

Such distinct vein tissues, as found in the corolla of *Petunia*, are not recognized in 'Tamanoura' petals. We often observe that the white marginal picotee in 'Tamanoura' petals tends to reduce or disappear at late bloom and on young plants (Savige, 1993), but the width of the white picotee part equally reduces against the edge of petals, not along the vein as in *petunia*. Although the suppression of *CHS* is likely to be caused by PTGS in the petals of 'Tamanoura' as well as in *petunia*, 'Tamanoura' seems to have a different controlling system to determine the boundary of red and white, depending on the environment and age.

Another possible explanation is the involvement of DNA methylation, which is an epigenetic regulation system to control gene expression (Richards, 1997). It is reported that the methylation state is affected by environmental conditions. Transposon Tam3 in *Antirrhinum majus* has a great influence on flower coloration (Hashida et al., 2003). The methylation level within Tam3 was high in higher temperature conditions (25°C), whereas reduced in lower temperature conditions (15°C). The hypermethylation state hinders the interaction of Tam3 *TPase* and stabilizes the transposition frequency of Tam3, therefore altering the expression of the gene into which Tam3 is inserted. If the white picotee formation of 'Tamanoura' depends on DNA methylation, some environmental factors might affect it by controlling the DNA methylation state during petal development. It would be of interest to compare the DNA methylation level of the *CjCHS* gene with the stage of floral bud development as well as to detect the accumulation of siRNA, causing PTGS, in the white picotee part.

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