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## Molecular Cloning of the Genes Involved in Anthocyanin Biosynthesis in *Camellia japonica*

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Partial cDNA sequences of three anthocyanin biosynthetic genes (*F3H*, *flavanone 3-hydroxylase*; *DFR*, *dihydroflavonol 4-reductase*; *ANS*, *anthocyanidin synthase*) were isolated from the petals of *Camellia japonica*. Their deduced partial amino acid sequences shared high homologies with those of woody plant species (CjF3Ha, 98.0%, CjF3Hb, 91.2% and CjDFR, 99.0% with *Camellia sinensis*; CjANS, 90.3% with *Rhododendron × pulchrum*). Some important amino acid residues for enzymatic activities were also conserved in the isolated clones, suggesting that the genes we identified in this study were the homologues of *C. japonica*. Gene-specific primer pairs were designed based on each partial cDNA sequence. The application of these primer pairs to RT-PCR analyses was tested.

### INTRODUCTION

The primary reason of the bright flower colours of plants is to be a signal to attract insects and birds for successful pollination by emphasising their floral tissue against a background of vegetation (Glover, 2007). The major pigments that cause flower colour are carotenoids, flavonoids and betalains (Davies, 2009). Flavonoids also have some additional important functions, like defence against pathogens and predators, prevention from damaging by UV light, action as antioxidants, and involvement in pollen development and germination (Glover, 2007). Anthocyanins are one of the plant-derived flavonoid compounds and are responsible for colours ranging from pink and red to purple and deep blue (Deroles, 2009). Most detailed studies about anthocyanin biosynthesis have been achieved in *Antirrhinum* and *Petunia* as models and many genes involved in the biosynthetic pathway have been isolated so far (Martin *et al.*, 1991; Holton and Cornish, 1995).

*Camellia japonica* has a long history as a representative woody ornamental plant in Japan (Tuyama, 1968a). Wild type *C. japonica* has a single-petalled red flowers, and two major anthocyanins were identified in the petals, namely cyanidin 3-glucoside and cyanidin 3-galactoside (Sakata *et al.*, 1986, 1987). However, molecular mechanism dominating their flower pigmentation has not been reported, and the breeding program of camellia plants still depends on the selection of the seedlings that appear by chance. Molecular information controlling flower characters of camellia must contribute to an efficient breeding system of the plant.

In this paper, we isolated partial cDNA sequences of three anthocyanin biosynthetic genes in *C. japonica*, *flavanone 3-hydroxylase* (*F3H*), *dihydroflavonol 4-reductase* (*DFR*) and *anthocyanidin synthase* (*ANS*), and confirmed that their gene-specific primer pairs are available for RT-PCR analyses.

### MATERIALS AND METHODS

#### Plant materials

Fully expanded flowers of wild *C. japonica* accession (collected in Kurose, Fukue Island, Nagasaki Prefecture, Japan), ‘Tamanoura’ and ‘Hatsu-arashi’ were picked immediately at the beginning of anthesis. The petals of ‘Tamanoura’ were separated into red and white marginal picotee parts, and only the red parts were used in this study. The petals were frozen promptly using liquid nitrogen and stored at –80°C until use for RNA extraction.

#### RNA extraction

RNA extraction was carried out following Kiefer *et al.* (2000)’s manner with some modifications. Frozen tissues (100–150 mg) were ground to fine powder with mortar and pestle using liquid nitrogen and 800 µl 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)] were added followed by incubation at 65°C for 10 min. One hundred µl of Nucleon PhytoPure DNA extraction resin (GE Healthcare, England) and 400 µl of 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 × g for 10 min at 4°C, aqueous phase was washed with 500 µl of chloroform/isoamylalcohol (24:1) at least three times. The aqueous phase, to which 500 µl of isopropanol were added, were incubated on ice for 1 h, followed by centrifugation at 9,000 × g for 10 min at 4°C. RNA pellets were dissolved in 100 µl DEPC water with

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2 M of 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, Germany) at 37 °C for 20 min. Equal volume of isopropanol were added for RNA precipitation, followed by centrifugation at  $9,000 \times g$  for 2 min at 4 °C. After two times wash with 70% EtOH, the pellets were dried and dissolved in DEPC water. The RNA concentration and purity were evaluated

with the absorbance at 260 nm.

### RT-PCR analyses

cDNA synthesis and RT-PCR were carried out using TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (Takara, Japan) following the manufacturer's instructions. We mixed two kinds of primers in the ratio of Oligo dT-adaptor primer:Random 9 mers = 4:1 and used for reverse tran-

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

Primer	Gene		Sequence
Degenerate primer	<i>F3H</i>	Forward	5'-ATG TCC GGT GGB AAR AAR GG-3'
		Reverse	5'-TTG CTC ATC TTC YTC YKG TAC-3'
	<i>DFR</i>	Forward	5'-RAG GAY CCY GAG AAT GAR G-3'
		Reverse	5'-GCT GTA YTT GAA YTY GAA YCC-3'
	<i>ANS</i>	Forward	5'-TTG AGT GGS AGG ATT AYT TYT TYC-3'
		Reverse	5'-GGT TCG CAG AAV ATH GCC C-3'
Gene-specific primer	<i>CjF3Ha</i>	Forward	5'-ACG GAG ACC TAC AGC GAG AA-3'
		Reverse	5'-ATG ATC CGC ATT CTT GAA CC-3'
	<i>CjDFR</i>	Forward	5'-AAC AAC CCA TTT TCG ACG AG-3'
		Reverse	5'-TTG TAC TCG GGC CAT TTC TC-3'
	<i>CjANS</i>	Forward	5'-ACG CAA AGC AAC TAC GAG G-3'
		Reverse	5'-CTA CCG TGT CAC CAA TGT GC-3'

B=C+G+T; R=A+G; Y=C+T; K=G+T; S=C+G; V=A+C+G; H=A+C+T

10	20	30	40	50	60
TGGATTCATC	GTTTCCAGTC	ATCTCCAGGG	AGAAGCAGTG	CAAGACTGGA	GAGAAATAGT
70	80	90	100	110	120
GACCTACTTC	TCATACCCGA	TCCGGGCCCG	GGACTATTCA	AGATGGCCCG	ACAAGCCCGA
130	140	150	160	170	180
AGGGTGGAGG	GCTGTGACGG	AGACCTACAG	CGAGAAATTG	ATGGACTTGG	CTTGCAAGTT
<b><i>CjF3Ha F</i></b>					
190	200	210	220	230	240
GCTGGAGGTG	TTGTCTGAGG	CCATGGACCT	TGAGAAGGAG	GCTCTTACAA	AAGCCTGTGT
250	260	270	280	290	300
TGATATGGAT	CAGAAGGTGG	TTGTAAATTT	CTACCCGAAA	TGCCCAAC	CCGACCTCAC
310	320	330	340	350	360
GCTCGGACTC	AAGCGACACA	CGGATCCGGG	TTCCATCACC	CTGCTCCTCC	AGGACCAGGT
370	380	390	400	410	420
TGGTGGGCTC	CAGGCCACTA	GAGATGGGGG	CAAGACCTGG	ATCACGGTTC	AGCCCGTGGA
430	440	450	460	470	480
GGGAGCTTTT	GTTGTTAATC	TGGGTGACCA	TGGTCATTAT	CTAAGCAATG	GGAGGTTCAA
490	500	510	520	530	540
GAATGCGGAT	CATCAGGCAG	TAGTGAATC	CAACTGCAGC	CGACTATCAA	TCGCTACATT
<b><i>CjF3Ha R</i></b>					
550	560	570	580	590	600
CCAGAACCCA	GCTCCCAGG	CGACAGTATA	CCCACTGAAG	ATTAGGGAGG	GAGAGAAGCC
610	620	630	640		
GGTTCTTGAA	GAGCCAATCA	CGTTCGCCGA	TAT.....		

**Fig. 1.** Nucleotide sequence of partial cDNA of *CjF3Ha*. The shadowed boxes represent the primer regions of *CjF3Ha F* and *R* used in this study.

scriptional reaction. PCR amplification was performed in a total volume of 50  $\mu$ l containing 45 ng template cDNA, 0.2  $\mu$ M of each primer, 10  $\mu$ l of 5  $\times$  PCR Buffer, 0.2 mM of each dNTP, 1.25 Unit *TaKaRa Ex Taq* HS polymerase. In case of using degenerate primer pairs, their volumes were calculated according to its degeneracy (Table 1). Finally, RNase Free H<sub>2</sub>O was added up to 50  $\mu$ l. Amplification was conducted using TaKaRa PCR Thermal Cycler Dice TP-600 (TaKaRa, Japan) with one cycle of 3 min at 94 °C, followed by 30 cycles of 20 sec at 94 °C, 20 sec at 56 °C and 1 min at 72 °C, finally one cycle of 10 min at 72 °C. PCR products were separated by electrophoresis in 1.5% (w/v) agarose gels and visualised under UV illumination after staining with ethidium bromide.

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

Degenerate RT-PCR products from wild *C. japonica* were subcloned into pGEM-T Easy Vector (Promega, USA) and transformed into Competent high *Escherichia coli* DH5  $\alpha$  (Toyobo, Japan). After culture on the LB

plates containing 100  $\mu$ g/ml of ampicillin, 100  $\mu$ g/ml of X-Gal and 23.83  $\mu$ g/ml of IPTG, only white colonies were selected, and then the plasmids containing inserts were extracted using LaboPass Plasmid Mini Purification Kit (Hokkaido System Science, Japan). BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA) and ABI PRISM 310 genetic analyzer (Applied Biosystems, USA) were employed for sequence analyses. The deduced amino acid sequence alignments and phylogenetic trees were obtained by using DNASIS-Mac v3.2 (Hitachi Software Engineering, Japan) and PHYLIP version 3.6 (Felsenstein, 2004), respectively.

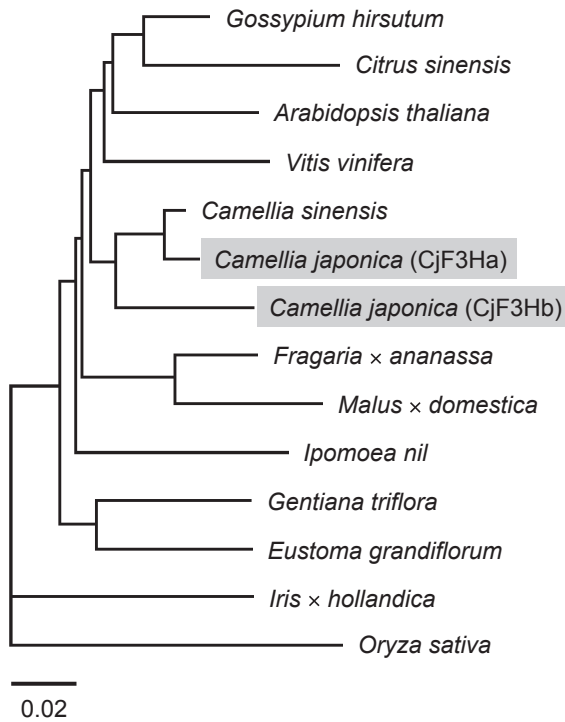
## RESULTS

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

We identified 633 bp fragment of partial *CjF3H*. Sequence analysis revealed that it contained multi-gene family. The major one was named *CjF3Ha* (GenBank accession no. AB524883) and minor one was *CjF3Hb*

		10	20	30	40	50	
CjF3Ha	1	GFIVSSHLQG	EAVQDWREIV	TYFSYPIRAR	DYSRWDPKPE	GWRAVTETYS	50
CjF3Hb	1	GFIVSSHLQG	EAVQDWREIV	TYFSYPIRAR	DYSRWDPKPE	EWRAVTEKYS	50
Vitis	1	GFIVSSHLQG	EAVQDWREIV	TYFSYPLRTR	DYSRWDPKPE	GWRSVTQEYS	50
Gentiana	1	GFIVSSHLQG	EAVRDWREIV	TYFSYPIKSR	DYSRWDPKPE	GWKSVTEKYS	50
Ipomoea nil	1	GFIVSSHLQG	EAVKDWREIV	TYFSYPVRAR	DYSRWDPKPE	GWRAVTEKYS	50
Arabidopsis	1	GFIVSSHLQG	EAVQDWREIV	TYFSYPVRNR	DYSRWDPKPE	GWVKVTEEYS	50
Oryza sativa	1	GFIVSSHLQG	EAVKDWREIV	TYFSYPVKSR	DYSRWDPKPA	GWRAVVEQYS	50
		*****	***	*****	*****	* * *	
		60	70	80	90	100	
CjF3Ha	51	EKLMDLACKL	LEVLSEAMD	EKEALTKACV	DMDQKVNVN	YPKCPQPDLT	100
CjF3Hb	51	SSLMEACKL	LEVLSEAMGL	EKEALTNACV	DMDQKVNVN	YPKCPQPDLT	100
Vitis	51	EKLMDLACKL	LEVLSEAMD	DKDALTNACV	DMDQKVNVN	YPQCPQPDLT	100
Gentiana	51	EQLMNLACKL	LEVLSESMRL	EKEALTKACV	DMDQKIVNVN	YPKCPQPDLT	100
Ipomoea nil	51	EKLMDLACKL	LEVLSEAMGL	EKEALSKACV	ELDQKLVNVN	YPKCPQPDLT	100
Arabidopsis	51	ERLMSLACKL	LEVLSEAMGL	EKESLTNACV	DMDQKIVNVN	YPKCPQPDLT	100
Oryza sativa	51	ERLMDLACKL	LGVLEAMGL	DTNALADACV	DMDQKVNVN	YPKCPQPDLT	100
		**	*****	* * *	*****	* * *	
		110	120	130	140	150	
CjF3Ha	101	LGLKRHTDPG	SITLLLDQDV	GGLQATRDGG	KTWITVQPV	GAFFVNLGDH	150
CjF3Hb	101	LGLKRHTDPG	TITLLLDQDV	GGLQATRDGG	KTWITVQPV	GAFFVNLGDH	150
Vitis	101	LGLKRHTDPG	TITLLLDQDV	GGLQATRDGG	KTWITVQPV	GAFFVNLGDH	150
Gentiana	101	LGLKRHTDPG	TITLLLDQDV	GGLQATRDGG	KSWITVQPV	GAFFVNLGDH	150
Ipomoea nil	101	LGLKRHTDPG	TITLLLDQDV	GGLQATRDGG	KTWITVQPV	GAFFVNLGDH	150
Arabidopsis	101	LGLKRHTDPG	TITLLLDQDV	GGLQATRDNG	KTWITVQPV	GAFFVNLGDH	150
Oryza sativa	101	LGLKRHTDPG	TITLLLDQDV	GGLQATRDAG	KTWITVQPI	GSFFVNLGDH	150
		*****	*****	*****	*****	*****	
		160	170	180	190	200	
CjF3Ha	151	GH-----	---YLSNGRF	KNADHQAVVN	SNCSRLSIAT	FQNPAPPEATV	200
CjF3Hb	151	GH-----	---YLSNGRF	KNADHQAVVN	SNSSRLSIAT	FQNPAPPEAIV	200
Vitis	151	GH-----	---YLSNGRF	KNADHQAVVN	SNSSRLSIAT	FQNPAPPEATV	200
Gentiana	151	GH-----	---YLSNGRF	KNADHQAVVN	SNYSRLSIAT	FQNPAPPEATV	200
Ipomoea nil	151	GH-----	---FLSNGRF	KNADHQAVVN	SEHSRMSIAT	FQNPAPPEAKV	200
Arabidopsis	151	GH-----	---FLSNGRF	KNADHQAVVN	SNSSRLSIAT	FQNPAPDATV	200
Oryza sativa	151	AHIMHLLGNV	NLQYLSNGRF	KNADHQAVVN	SDCCRLSIAT	FQNPAPDAMV	200
		*	*****	*****	*****	*****	
		210	220	230	240	250	
CjF3Ha	201	YPLKIREGEK	PVLEEPITFA	D.....	.....	.....	250
CjF3Hb	201	YPLKIREGEK	SIMEEPITFP	D.....	.....	.....	250
Vitis	201	YPLKIREGEK	AVLEGPITFA	E.....	.....	.....	250
Gentiana	201	YPLAIRDGEK	PVLDEPITFA	E.....	.....	.....	250
Ipomoea nil	201	YPLKVREGEK	PILEEPITFA	E.....	.....	.....	250
Arabidopsis	201	YPLKVREGEK	AILEEPITFA	E.....	.....	.....	250
Oryza sativa	201	YPLAVRDGEE	PILEEPITFA	E.....	.....	.....	250
		***	***	****			

**Fig. 2.** Comparison of the deduced amino acid sequences of partial *CjF3Hs* and those of corresponding parts of other plant species. Asterisks indicate completely identical amino acid residues among six species. The shadowed amino acid residues (His106, Asp108 and His175) represent absolute conservation important for catalytic activity. Species names and their GenBank accession numbers are as follows; *Arabidopsis thaliana* (AF064064); *Gentiana triflora* (AB193311); *Ipomoea nil* (D83041); *Oryza sativa* (*Oryza sativa*, NM001060692); *Vitis vinifera* (EF192467).



**Fig. 3.** A phylogenetic tree for F3H constructed by neighbor-joining methods using partial deduced amino acid sequences, including CjF3Ha (AB524883), CjF3Hb (AB524884) identified in this study. Sequence data were collected from GenBank databases [*Arabidopsis thaliana* (AF064064), *Camellia sinensis* (AY641730), *Citrus sinensis* (AB011795), *Eustoma grandiflorum* (AB078956), *Fragaria x ananassa* (AB201760), *Gentiana triflora* (AB193311), *Gossypium hirsutum* (EF187440), *Ipomoea nil* (D83041), *Iris x hollandica* (AB183826), *Malus x domestica* (AB074486), *Oryza sativa* (NM001060692), *Vitis vinifera* (EF192467)].

10	20	30	40	50	60
TAATCAAGCC	GACAATCAAC	GGTGTGTTGA	GCATCATAAG	GTCATGCACC	AAAGCTAAGA
70	80	90	100	110	120
CAGTGAAGAG	GCTGGTGTTC	ACATCCTCTG	CTGGAACGTG	TAATGTCCAG	GAACACCAAC
130	140	150	160	170	180
AAACCATTTT	CGACGAGAAC	AATTGGAGTG	ACTTGGATTG	CATCAATAAG	AAGAAGATGA
<b>CjDFR F</b>					
190	200	210	220	230	240
CTGGCTGGAT	GTATTTTGTG	TCAAAAACAT	TGGCAGAGAA	AGCAGCATGG	GAAGCAGCAA
250	260	270	280	290	300
AAGAGAACAA	CATTGATTTC	ATTAGTATCA	TTCCTACATT	AGTTGTAGGA	CCTTTCATCA
310	320	330	340	350	360
TGCCAACATT	CCCACCAAGC	CTAATCACCG	CTCTCTCCCC	CATCACTAGG	AATGAAGGAC
370	380	390	400	410	420
ACTATTCGAT	CATAAAGCAA	GGGCAGTTTG	TGCACCTTGA	TGATCTCTGT	GAATCTCATA
430	440	450	460	470	480
TATTCTTGTA	TGAGCATCCT	CAGGCTGAGG	GCAGATACAT	TTGCTCCTCC	CATGATGCTA
490	500	510	520	530	540
CCATCCATGA	TTTGGCCAAA	CTGATGAGAG	AGAAATGGCC	CGAGTACAAAT	GTCCCCACTG
<b>CjDFR R</b>					
550	560	570	580	590	600
AGTTTAAGGG	GATAGACAAG	GACTTGCCAG	TTGTGTCGTT	CTCATCGAAG	AAGTTGATAG
610	GAATG.....				

**Fig. 4.** Nucleotide sequence of partial cDNA of *CjDFR*. The shadowed boxes represent the primer regions of *CjDFR* F and R used in this study.

		10	20	30	40	50	
CjDFR	1	IKPTINGVLS	IIRSCAKT	VKRLVFTSSA	GTNVNQEHOQ	PIFDENNWS	50
Vitis	1	IKPTIEGMLG	IMKSCAAKT	VRRLVFTSSA	GTVNIQEHQL	PVYDESCWSD	50
Malus	1	IKPTINGLLD	ILKACQKAKT	VRRLVFTSSA	GTNVNVEEHQK	PVYDESNNWS	50
Rosa	1	IKPTINGVLD	IMQACLKAKT	VRRLVFTSSA	GSVNVEETQK	PVYNESNNWS	50
Gentiana	1	IKPTIDGFLS	IIRSCVAKT	VKRLVFTSSA	GTVDVQEQQK	PVYDENDWSD	50
Ipomoea nil	1	IKPAINGVLN	IINSCVAKT	VKRLVFTSSA	GTLNVQPQQK	PVYDETCWSD	50
Arabidopsis	1	IKPTVNGMLG	IMKACVKAKT	VRRFVFTSSA	GTNVNVEEHQK	NVYDENDWSD	50
Oryza sativa	1	VKPTVEGMLS	IMRACRDAGT	VKRIVFTSSA	GTVNIEERQR	PSYDHDDWSD	50
		** * *	* * *	*****	*	***	
		60	70	80	90	100	
CjDFR	51	LDFINKKMT	GWMYFVSKTL	AEKAAWEAAK	ENNIDFISII	PTLVVGPFFIM	100
Vitis	51	MEFCRAKMT	AWMYFVSKTL	AEQAANKYAK	ENNIDFITII	PTLVVGPFFIM	100
Malus	51	VEFCRSVKMT	GWMYFVSKTL	AEQAANKYAK	ENNIDFITII	PTLVIGPFFIM	100
Rosa	51	VEFCRRVKMT	GWMYFASKTL	AEQEAANKFAK	ENNIDFITII	PTLVIGPFFIM	100
Gentiana	51	LDFINSTKMT	GWMYFVSKIL	AEKAAWEVTK	ANDIGFISII	PTLVVGPFFIT	100
Ipomoea nil	51	LDFIYAKMT	GWMYFASKIL	AEKEANKVTK	EKKIDFISII	PPLVVGPFIT	100
Arabidopsis	51	LEFIMSKMT	GWMYFVSKSL	AEKAAWDAE	EKGLDFISII	PTLVVGPFIT	100
Oryza sativa	51	IDFCRRVKMT	GWMYFVSKSL	AEKAAMEYAR	EHGLDLISVI	PTLVVGPFFIS	100
		* ***	**** *	*** *	*	*** **	
		110	120	130	140	150	
CjDFR	101	PTFPSSLITA	LSPITRNEGH	YSIIKQGQFV	HLDDLCESHI	FLYEHQAEG	150
Vitis	101	SSMPSSLITA	LSPITGNEAH	YSIIKQGQFV	HLDDLCNAHI	YLFENPKAEG	150
Malus	101	PSMPPSLITG	LSPILRNESH	YGIKQGQYV	HLDDLCLSHI	YLYEHPKAEG	150
Rosa	101	PSMPPSLITG	LSPLTGNEAH	YSIIKQGQFI	HLDDLCQSHI	YLYEHPKAEG	150
Gentiana	101	TTFPSSLITA	LSLITGNEAH	YGIKQGQFV	HLDDLCEAHI	FLYEHPEAEG	150
Ipomoea nil	101	PTFPSSLITA	LSLITGNQAH	YSIIKQGQYV	HLDDLCEAHI	FLYEHHPKAEG	150
Arabidopsis	101	TSMPPSSLITA	LSPITRNEAH	YSIIKQGQYV	HLDDLCNAHI	FLYEQAARKG	150
Oryza sativa	101	NGMPPSHVTA	LALLTGNEAH	YSILKQVQFV	HLDDLCDAEI	FLFESPEARG	150
		*** *	* * *	*** *	*****	* * *	
		160	170	180	190	200	
CjDFR	151	RYICSSHDAT	IHDLAKLMLRE	KWPEYNVPT	FKGID-KDLP	VVSFSSKKLI	200
Vitis	151	RYICSSHDCI	ILDLAKLMLRE	KYPEYNIPTE	FKGVD-ENLK	SVCFSKKLT	200
Malus	151	RYICSSHDAT	IHELVKMLRE	KYPEYNIPTK	FKGID-DNLE	PVHFSSKKLR	200
Rosa	151	RYICSSHDAT	IHEIAKLLKG	KYPEYNVPTT	FKGIE-ENLP	KVHFSSKKLL	200
Gentiana	151	RYICSSHDTT	IHDLAKMIRQ	NWPEYYIPTK	LKGID-EDIP	VVSFSSNKLI	200
Ipomoea nil	151	RFICSSHHTT	IHGLADMITQ	NWPEYYIPSE	FKGIE-KDLP	VVFSSKKLQ	200
Arabidopsis	151	RYICSSHDAT	ILTISKFLRP	KYPEYNVPT	FEGVD-ENLK	SIEFSSKKLT	200
Oryza sativa	151	RYVCSHDAT	IHGLATMLAD	MFPEYDVPRS	FPGIDADHLQ	PVHFSSWKLL	200
		* ****	*	*** *	*	*** **	
		210	220	230	240	250	
CjDFR	201	GM.....	.....	.....	.....	.....	250
Vitis	201	DL.....	.....	.....	.....	.....	250
Malus	201	EI.....	.....	.....	.....	.....	250
Rosa	201	ET.....	.....	.....	.....	.....	250
Gentiana	201	DL.....	.....	.....	.....	.....	250
Ipomoea nil	201	DM.....	.....	.....	.....	.....	250
Arabidopsis	201	EM.....	.....	.....	.....	.....	250
Oryza sativa	201	AH.....	.....	.....	.....	.....	250

**Fig. 5.** Comparison of the deduced amino acid sequence of partial *CjDFR* and those of corresponding parts of other plant species. Asterisks indicate completely identical amino acid residues among eight species. The shadowed amino acid residues (Ser29 and Tyr64) represent absolute conservation important for catalytic activity. Species names and their GenBank accession numbers are as follows; Arabidopsis (*Arabidopsis thaliana*, AB033294); Gentiana (*Gentiana triflora*, D85185); Ipomoea nil (*Ipomoea nil*, AB006792); Malus (*Malus × domestica*, AY227728); Oryza sativa (*Oryza sativa*, AB003496); Rosa (*Rosa hybrida*, D85102); Vitis (*Vitis vinifera*, X75964).

(GenBank accession no. AB524884), both encoded 210 amino acid residues (Figs. 1 and 2). Phylogenetic tree was constructed to compare the homology of deduced amino acid sequences of CjF3Ha and CjF3Hb with other related F3Hs (Fig. 3). CjF3Ha and CjF3Hb shared 98.0% and 91.2% identities with F3H from *Camellia sinensis*, respectively.

The length of partial *DFR* clone of *C. japonica* was 605 bp, in which 201 amino acid residues were encoded (Figs. 4 and 5) (GenBank accession no. AB524885). Figure 6 shows the comparison of deduced amino acid sequences of partial DFRs. The deduced amino acid sequence of CjDFR showed high identity with that of *Camellia sinensis* (99.0%).

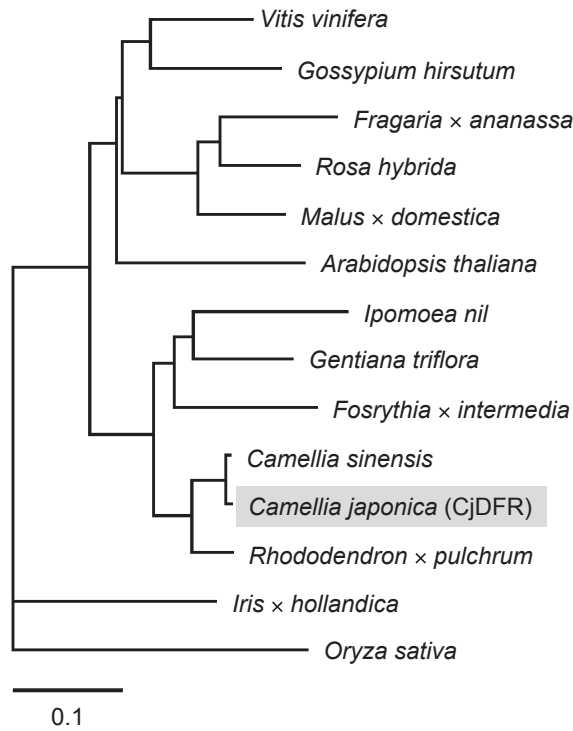
Partial *CjANS* length we obtained was 468 bp and it encoded 155 amino acid residues (Figs. 7 and 8)

(GenBank accession no. AB524886). The deduced amino acid sequence of CjANS clustered with those of woody plants, for instance, *Rhododendron × pulchrum* (90.3%) (Fig. 9).

#### Gene-specific RT-PCR

We designed gene-specific primer pairs for each gene (*CjF3Ha*, *CjDFR* and *CjANS*) based on the cDNA sequences we obtained in this study (Figs. 1, 4, 7 and Table 1). Although two sequences in *F3H*, *CjF3Ha* and *CjF3Hb*, were identified, *CjF3Ha* was applied for the design of *CjF3H* gene-specific primer, because of its frequent appearance in cloning procedure. Bands of expected sizes were amplified successfully in all samples, wild type *C. japonica* and 'Tamanoura' (red parts of the petals) and 'Hatsu-arashi' with RT-PCR using these





**Fig. 6.** A phylogenetic tree for DFR constructed by neighbor-joining methods using partial deduced amino acid sequences, including CjDFR (AB524885) identified in this study. Sequence data were collected from GenBank databases [*Arabidopsis thaliana* (AB033294), *Camellia sinensis* (AB018685), *Fosrythia x intermedia* (Y09127), *Fragaria x ananassa* (AY695812), *Gentiana triflora* (D85185), *Gossypium hirsutum* (EF187441), *Ipomoea nil* (AB006792), *Iris x hollandica* (AB304917), *Malus x domestica* (AY227728), *Oryza sativa* (AB003496), *Rhododendron x pulchrum* (AB289595), *Rosa hybrida* (D85102), *Vitis vinifera* (X75964)].

10	20	30	40	50	60
ACCTTGCTCTT	CCCTGAAGAC	AAGCGTGACA	TGTCCATTTG	GCCTAAGACA	CCATCCGACT
70	80	90	100	110	120
ATATTCCGGC	AACAAGCGAG	TACGCAAAGC	AACTACGAGG	TCTAGCAACA	AAAGTCCTGT
<i>CjANS F</i>					
130	140	150	160	170	180
CGGCCCTCTC	ACTCGGCTTG	GGACTIONAAG	AAGGCCGACT	AGAAAAAGAA	GTAGGAGGCA
190	200	210	220	230	240
TGGAAGAGCT	GCATCTCCAA	ATGAAAATAA	ACTATTACCC	AAAATGCCCT	CAGCCAGAGC
250	260	270	280	290	300
TCGCCCTCGG	CGTCGAAGCC	CACACCGACG	TCTCTGCCCT	CACCTTCATC	CTCCACAACA
310	320	330	340	350	360
TGGTTCCCGG	CCTGCAACTC	TTCTACGAGG	GCAAATGGAT	CACTGCCAAA	TGCGTCCCCA
370	380	390	400	410	420
ACTCCATTAT	CATGCACATT	GGTGACACGG	TAGAAATTCT	CAGTAACCGC	AAGTACAAGA
<i>CjANS R</i>					
430	440	450	460	470	
GCATTCTCCA	TCGTGGACTC	GTTAATAAAG	AAAAAGTGAG	GATTTCGT	..

**Fig. 7.** Nucleotide sequence of partial cDNA of *CjANS*. The shadowed boxes represent the primer regions of *CjANS F* and *R* used in this study.

primer pairs (*CjF3Ha*, 357bp; *CjDFR*, 412bp; *CjANS*, 312bp) (Fig. 10).

## DISCUSSION

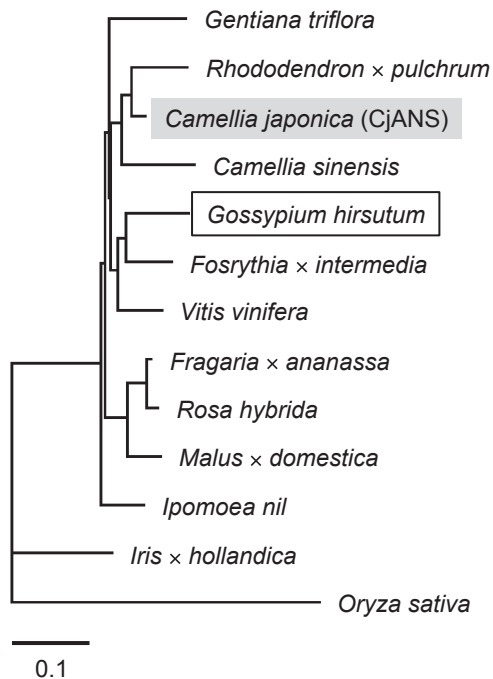
We identified the partial cDNA sequences of *CjF3H*, *CjDFR* and *CjANS*. Their deduced amino acid sequences showed high identities with those of woody plants (Figs.

3, 6 and 9) and some amino acid residues important for their catalytic activities were conserved in the sequences obtained in this study (Figs. 2, 5 and 8). These facts support that the genes we identified were the homologues of *C. japonica*.

Gene-specific primer pairs were constructed to amplify the parts of three anthocyanin biosynthetic genes, *CjF3Ha*, *CjDFR* and *CjANS* (Figs. 1, 4, 7 and Table 1).

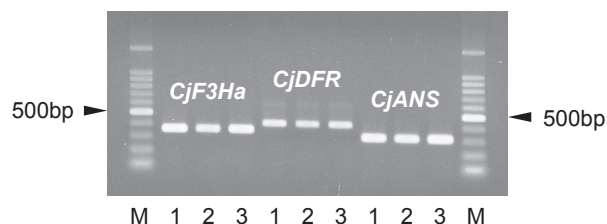
		10	20	30	40	50	
CjANS	1	LVFPEDKRD	SIWPKTPSDY	IPATSEYAKQ	LRGLATKVLS	ALSLGLGLEE	50
Vitis	1	LIFPEDKRD	TIWPKTPSDY	VPATCEYSVK	LRSLATKILS	VLSLGLGLEE	50
Malus	1	CVYPEDKRD	SIWPKTPADY	TEATAEYAKQ	LRELATKVLS	VLSLGLGLDE	50
Rosa	1	CVYPEDKRD	SIWPKTPSDY	IVATSEYAKE	LRGLATKILT	VLSLGLGLEE	50
Gentiana	1	CIYPERKRD	SIWPKTPHDY	IPATIEYAKQ	LRDLATKVLA	VLSVGLGLEP	50
Ipomoea nil	1	CIFPEDKTDL	SIWPKTPSDY	IDATKEYAKQ	LRALATKVLA	VLSLGLGLEE	50
Arabidopsis	1	NYLPSISIRNP	SKWPSQPPKI	RELIEKYGEE	VRKLCERLLE	TLSESLGLKP	50
Oryza sativa	1	LVHPDHLADH	SLWPANPPEY	VPVSRDFGGR	VRTLASKLLA	ILSLGLGLPE	50
		*	** *		* *	** **	
		60	70	80	90	100	
CjANS	51	GRLEK----	EVGGMEE-LH	LQMKINYYPK	CPQPELALGV	EAHTDVSALT	100
Vitis	51	GRLEK----	EVGGMEE-LI	LQKKINYYPK	CPQPELALGV	EAHTDVSALT	100
Malus	51	GRLEK----	EVGGLEE-LI	LQMKINYYPK	CPQPELALGV	EAHTDVSALT	100
Rosa	51	GRLEK----	EVGGLEE-LV	LQMKINYYPK	CPQPELALGV	EAHTDISALT	100
Gentiana	51	DRLEN----	EVGGMEE-MI	LQKKINYYPK	CPQPELALGV	EAHTDVSALT	100
Ipomoea nil	51	GRLEK----	EVGGMEE-LI	LQMKINYYPK	CPQPELALGV	EAHTDVSALT	100
Arabidopsis	51	NKLMQALGGG	DKVGAS----	--LRTNFYPK	CPQPQLTLGL	SSHSDPGGIT	100
Oryza sativa	51	ETLERRLRGH	ELAGVDDDLL	LQLKINYYPR	CPRPDLAVGV	EAHTDVSALS	100
		*	*	***	*** ** *	** *	
		110	120	130	140	150	
CjANS	101	FILHN-MVPG	LQLFYEGKWI	TAKCVNSII	MHIGDTVEIL	SNRKYKSILH	150
Vitis	101	FILHN-MVPG	LQLFYEGKWI	TAKCVNSII	MHIGDTIEIL	SNGKYKSILH	150
Malus	101	FILHN-MVPG	LQLFYEGKWI	TAKCVNSIV	MHIGDTLEIL	SNGKYKSILH	150
Rosa	101	FILHN-MVPG	LQLFYGGKWI	TAKCVNSIV	MHIGDTLEIL	SNGKYKSILH	150
Gentiana	101	FILHN-MVPG	LQLFYQGWV	TAKCVNSII	MHVGDTEIL	SNGKYKSILH	150
Ipomoea nil	101	FILHN-MVPG	LQLFYGGKWI	TAKCVNSII	MHVGDTEIL	SNGKYKSILH	150
Arabidopsis	101	ILLPDEKVG	LQVRRGDGWV	TIKSVPNALI	VNIGDQLQIL	SNGIYKSVEH	150
Oryza sativa	101	FILHN-GVPG	LQVHHAGSWV	TARPEPGTIV	VHVGDLEIL	TNGRYTSVLH	150
		*	* ** *	*	*	** ** *	
		160	170	180	190	200	
CjANS	151	RGLVNKEKVR	IS.....	.....	.....	.....	200
Vitis	151	RGLVNKEKVR	IS.....	.....	.....	.....	200
Malus	151	RGMVNKEKVR	IS.....	.....	.....	.....	200
Rosa	151	RGLVNKEKVR	IS.....	.....	.....	.....	200
Gentiana	151	RGLVNKEKVR	IS.....	.....	.....	.....	200
Ipomoea nil	151	RGVVNREKVR	VS.....	.....	.....	.....	200
Arabidopsis	151	QVIVNSGMR	VS.....	.....	.....	.....	200
Oryza sativa	151	RGLVSRDAVR	LS.....	.....	.....	.....	200
		*	* *				

**Fig. 8.** Comparison of the deduced amino acid sequence of partial *CjANS* and those of corresponding parts of other plant species. Asterisks indicate completely identical amino acid residues among eight species. The shadowed amino acid residues (His93, Asp95 and His150) represent absolute conservation important for catalytic activity. Species names and their GenBank accession numbers are as follows; Arabidopsis (*Arabidopsis thaliana*, AY093302); Gentiana (*Gentiana triflora*, AB193310); Ipomoea nil (*Ipomoea nil*, AB073920); Malus (*Malus × domestica*, AB074487); Oryza sativa (*Oryza sativa*, Y07955); Rosa (*Rosa hybrida*, AB239787); Vitis (*Vitis vinifera*, EF192468).



**Fig. 9.** A phylogenetic tree for ANS constructed by neighbor-joining methods using partial deduced amino acid sequences, including *CjANS* (AB524886) identified in this study. Sequence data were collected from GenBank databases [*Camellia sinensis* (AY830416), *Fosrythia × intermedia* (Y12489), *Fragaria × ananassa* (AY695817), *Gentiana triflora* (AB193310), *Gossypium hirsutum* (EF187442), *Ipomoea nil* (AB073920), *Iris × hollandica* (AB284174), *Malus × domestica* (AB074487), *Oryza sativa* (Y07955), *Rhododendron × pulchrum* (AB289596), *Rosa hybrida* (AB239787), *Vitis vinifera* (EF192468)].





**Fig. 10.** RT-PCR products of *CjF3Ha*, *CjDFR* and *CjANS* using gene-specific primer pairs designed in this study. M indicates 100 bp DNA Ladder Marker. 1, wild type *C. japonica*; 2, 'Tamanoura'; 3, 'Hatsu-arashi'. Three independent flowers were analysed per each accession.

They successfully amplified single PCR products of expected sizes (Fig. 10). It was, therefore, shown that primer pairs designed here are applicable to investigate the expression profiles of these genes on petal pigmentation of *C. japonica*.

Insertion or excision of transposable elements on anthocyanin biosynthetic genes caused flower colour mutation, like fleck and sector in the genus *Ipomoea* (Iida *et al.*, 2004). *Camellia japonica* has been domesticated for a long time in Japan (Tuyama, 1968a), and there are many cultivars bearing flowers which imply such involvements of genetic mutations (Tuyama, 1968b). Molecular information we presented here is useful to elucidate the mechanism of such flower colour mutation in *C. japonica*.

*Camellia japonica* 'Hatsu-arashi' lacks an accumulation of anthocyanins and results in generating a white coloured flower (Savidge, 1993). However, the expression levels of three genes tested in its petals showed no differences from those of wild type *C. japonica* and 'Tamanoura' (red parts of the petals) (Fig. 10). One possibility is that other anthocyanin biosynthetic genes except *CjF3H*, *CjDFR* and *CjANS* might be down-regulated. An alternative is an involvement of mutations caused by nucleotide substitution or insertion/deletion, if some of the above three genes are responsible. These mutations induce an amino acid substitution or frame shift translation, which are responsible for loss of enzymatic activities, although mRNA transcriptional levels are not suppressed, as in the case of *Matthiola incana* (Hemleben *et al.*, 2004).

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