ORIGINAL RESEARCH

Molecular Analysis of Anthocyanin Biosynthetic Genes and Control of Flower Coloration by Flavonoid 3',5'-Hydroxylase (F3'5'H) in *Dendrobium moniliforme*

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Abstract *Dendrobium moniliforme* is a native species of Korea. The flower of this species is composed of a reproductive column and white perianths including petals, sepals and lip, but the base of the column bears reddish purple pigment spots. Anthocyanins are major pigments that contribute flower color in *Dendrobium*. Three key anthocyanin biosynthetic genes encoding dihydroflavonol 4-reductase (DFR), chalcone synthase (CHS), and flavonoid 3',5'-hydroxylase (F3'5'H) were isolated and analyzed for their expression patterns in floral organs to understand the molecular mechanism underlying flower color development. Quantitative RT-PCR analysis revealed that *F3'5'H* transcripts were highly accumulated in the base of the column compared with those of perianths, but the other two genes showed no significant difference among the floral organs.

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K.-W. Kang Babo Orchid Farm, Gyenggi-do 472-831, South Korea Microprojectile bombardment using the white perianths revealed that the transient expression of F3'5'H gene, but not *DFR* and *CHS* genes, was sufficient to produce reddish purple colored pigmentation. These results suggest that the lack of colors in perianths of *D. moniliforme* is at least due to transcriptional control of F3'5'H. The data presented here may improve our understanding of the mechanisms underlying floral color development in *D. moniliforme* and contribute to advances in orchid biotechnology.

Keywords Dendrobium moniliforme · Anthocyanin ·

Flower color · Microprojectile bombardment · Flavonoid 3',5'-hydroxylase (F3'5'H)

Abbreviations

RT-PCR	Reverse transcription-polymerase chain reaction
CHS	Chalcone synthase
DFR	Dihydroflavonol 4-reductases
F3'5'H	Flavonoid 3',5'-hydroxylase
F3'H	Flavonoid 3'-hydroxylase
F3H	Flavanone 3-hydroxylase
ANS	Anthocyanidin synthase
DHK	Dihydrokaempferol
DHQ	Dihydroquercetin
DHM	Dihydromyricetin

Introduction

The genus *Dendrobium* is the second largest group of the family Orchidaceae, which comprises approximately 1,400 species worldwide (Dressler 1990; Wood 2006). Since species from the genus *Dendrobium* produce valuable floral

traits including colors, morphologies, and scents, they have been regarded as one of the most important commercial orchids that are used for cut flowers and potted plants in the floricultural industry (Yu et al. 2001). In particular, flower color has been regarded as an important characteristic attracting the attention of consumers. To improve flower colors for marketable qualities, a variety of *Dendrobium* hybrids have been created by a traditional breeding strategy through intraand inter-specific crosses. However, creation or modification of some flower colors is still difficult due to a limitation of gene resources in the species. With recent advances in tissue culture and genetic transformation technology in *Dendrobium*, it has now become feasible to develop specific desired flower colors in relatively short periods at a large scale (Nan and Kuehnle 1995; Chai and Yu 2007).

Many studies have demonstrated that chemical structures of anthocyanins, a class of flavonoids, are primarily responsible for the broad range of flower colors in the Dendrobium (Hahlbrock and Griesbach 1975: Holton and Cornish 1995; Winkel-Shirley 2001). Although hundreds of anthocyanins have been reported, they can be classified into three major types: cyaniding (red to magenta), delphinidin (purple to violet), and pelargonidin (brick red to scarlet). Moreover, anthocyanins change their colors depending on vacuolar pH, co-pigmentation, or the formation of a complex with metal ions (Kondo et al. 1992; Yoshida et al. 2003; Shiono et al. 2005; Shoji et al. 2007). The anthocyanin biosynthetic pathway that determines floral pigmentation is generally conserved among plant species, and genes that encode the enzymes regarding this pathway have been mostly isolated and extensively studied (Holton and Cornish 1995; reviewed in Tanaka and Ohmiya 2008).

Flower coloration is also thought to be specified by lack of specific genes that are implicated in anthocyanin biosynthesis (Holton and Cornish 1995; Tanaka and Ohmiya 2008). Thus, plant species usually exhibit a limited number of flower colors, and no species displays all the possible flower colors. For example, roses, carnations and chrysanthemum lack violet/blue varieties due to deficiency of flavonoid 3',5'-hydroxylase (F3'5'H), a key enzyme in the synthesis of delphinidin-based anthocyanins (Holton and Tanaka 1994). Petunia and Cymbidium lack red/orange varieties due to the absence of dihydroflavonol reductases (DFRs) specificity toward dihydrokaempferol (DHK) substrate, resulting in low accumulation of pelargonidin-based anthocyanins (Forkmann and Ruhnau 1987; Johnson et al. 1999). It was also reported that recessive mutations at the DFR gene of maize lead to a colorless aleurone layer (Reddy et al. 1987). CHS represents the first committed step in the anthocyanin pathway that catalyzes the formation of naringenin chalcone from malonyl-CoA and 4-coumaroyl-CoA (Tanaka and Ohmiya 2008). Insertional mutations of *CHS* by transposons resulted in an albino mutant phenotype in the *Ipomoea purpurea* and *I. nil* floral limb (Epperson and Clegg 1987; Habu et al. 1998).

Genetic studies have identified several transcription factors that affect the expression of multiple anthocyanin structural genes in Arabidopsis, maize, snapdragon, Oncidium and petunia (Broun 2004; Koes et al. 2005; Quattrocchio et al. 1999; Chiou and Yeh 2008). They are known to belong to three distinct gene families including R2R3-MYB, basic helix-loop-helix, and WD40 repeats. Moreover, other regulatory genes encoding WRKY transcription factor (Johnson et al. 2002), zinc finger protein (Sagasser et al. 2002), MADS domain protein (Nesi et al. 2002), and homeodomain protein (Kubo et al. 1999), have been reported to involve anthocyanin biosynthesis as well as other aspects of plant development. However, their regulatory mechanism is not fully understood since the expression of anthocyanin structural genes is differentially modulated by a regulatory complex composed of different types of transcription factor (Martin and Gerats 1993; Koes et al. 2005).

In this study, we isolated three key anthocyanin biosynthetic genes and analyzed their possible functions with the aims of understanding the molecular mechanism underlying flower pigmentation in *Dendrobium moniliforme*. Our data presented here may be useful for the genetic manipulation of flower pigmentation in the future.

Materials and Methods

Plant Materials and Growth Condition

D. moniliforme was collected from Jindo Island in Korea and cultivated in the greenhouse facility of Chonbuk National University. *D. moniliforme* seeds originated from a single plant were grown on MS agar medium containing 2% peptone for 1 year and then transferred to the greenhouse to produce flowers. Flower buds were harvested just before or after flowering in late May and throughout June. The harvested flower buds were then dissected into petal, sepal, lip and the column including the base, and each organ was combined and kept at -70° C until used. Total RNA was extracted from the combined samples.

Total RNA Isolation and RT-PCR Analysis

Total RNA was isolated using Trizol reagent (Gibco BRL, NY, USA) and then treated with RQ1-DNase I (Promega, Germany) to remove DNA contamination, according to the manufacturer instructions. To examine the expression patterns of anthocyanin biosynthetic genes, total RNA

Target gene	Forward primer sequence (5' to 3')	Reverse primer sequence $(5' \text{ to } 3')$
Primers for cDNA	cloning	
CHS	AACCATGGCGCCSCCGGCAATGGAAGAG	TCACACCGCACCAGCAATCGGA
DFR	ATGGAGAATGAGAAGAAGGGWCCAGTAGTG	CACTTAACAGCAATCTGYTCTTTAACTTCC
F3′5′H	ATGTCYATCTTCCTCATCRCMWCMCTCYTC	TTAAASAASSCCATAMGCCGCCGSCGSC
Primers for RT-PC	R analysis	
CHS	TTCGCCGGCGGCACCGTCCT	AGCCTCCGCCGACCTCCGCC
DFR	GGCCGGCAGCGTTCAGCGAGTG	TGAGTGGGATAAGCTTCTTATCCC
F3′5′H	CGGGGACGGACACCTCCGCCAT	CGGCAACACCAACCCCGGCCCT
Actin	TGCTAGTGGCCGCACGACAGGT	GGGCACCTAAATCTCCCAGCTCC
Primers for quantit	tative RT-PCR analysis	
CHS	GAAAGACGTCCCAGGCTTGAT1	TGAATACCAAGCGGCTCGAA
DFR	AGGCTGCTTGGGAGTTTGTAAAG	AGGACCCCACCACCAAAGTT
F3′5′H	CATGGTCTTCGCCGATTACG	TTGGAGCCGAGGAGATGGA
Actin	AGCCGAGATCTCACAGACTCCTT	ACGCTCTGCAGTAGTGGTGAAAG

isolated from each floral organ was subjected to semiquantitative RT-PCR analysis as described previously (Park et al. 2010). PCR conditions were as follows: one cycle of 94°C for 3 min; 20–30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension period of 72°C for 10 min. To use as an internal control, a partial cDNA fragment of the *actin* gene was obtained by RT-PCR using the degenerate primers, 5'-GARAARATGACNCAR ATHATG-3' and 5'-TCNACRTCRCAYTTCATDAT-3'. After the PCR products were sequenced, primers specific for the *actin* gene were designed (Table 1). Expression levels were precisely quantified by quantitative RT-PCR analysis. One twentieth of the first-strand cDNA was mixed with an equal volume of $\times 2$ SYBR Green Master Mix (Applied Biosystems, USA) and the appropriate primers as listed in Table 1. PCR conditions were as follows: 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 1 min using the 7500 Real-Time PCR System (Applied Biosystems, USA). Results were analyzed with SDS 1.7 software (Applied Biosystems, USA). Relative expression levels of each gene are normalized to that of actin gene.

Isolation of Full-Length cDNA Clones

Three micrograms of total RNA isolated from the column of base was reverse transcribed using the Impron II Reverse



Fig. 1 Structure of *Dendrobium moniliforme* flower and anthocyanin biosynthetic pathway. **a** Whole plant phenotype of *D. moniliforme*. **b** Representation of different floral organs including three sepals, two petals, lip, column, and the base of the column. Lip was detached from the flower to show the base of the column. **c** Magnification image of the base of the column. *Scale bars* indicate 5 mm. **d** Proposed model

for anthocyanin biosynthetic pathway in orchid (Tanaka and Ohmiya 2008). Names of enzymes are abbreviated as follows: *CHS* chalcone synthase, *CHI* chalcone flavanone isomerase, *F3H* flavanone 3-hydroxylase, *F3'H* flavonoid 3'-hydroxylase, *F3'5'H* flavonoid 3',5'-hydroxylase, *DFR* dihydroflavonol 4-redutase, *ANS* anthocyanidin synthase

Transcription System (Promega) as described previously (Park et al. 2010). Then, one tenth of the first-strand cDNA samples were used as a template for RT-PCR using the degenerated primers as listed in Table 1. Degenerated primers were designed to amplify the full-length anthocyanin biosynthetic genes based on an alignment of 5' and 3'end sequence of orchid genes that are previously deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank): *chalcone synthase* (*CHS*; AM286424, AM490639, AY741319, DQ462460, FM209429, and FM209430), *dihydroflavonol 4-reductase* (*DFR*; AY741318, FJ426271, FM209431, and FM209432), and *flavonoid 3'*, 5'-hydroxylase (F3'5'H; DQ923127 and DQ148458). PCR conditions were as follows: one cycle of 94°C for 3 min, 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min and a final extension period of 72°C for 10 min. The resulting PCR products were recovered from agarose gel electrophoresis and cloned into a pBluescript-T vector as described previously (Park et al. 2010). All full-length clones were confirmed of their nucleotide sequence by DNA sequencing. GenBank accession numbers are HQ412558 (CHS), HQ412559 (DFR), and HQ412560 (F3'5'H). The BLASTn and BLASTp algorithm in the NCBI database (http://www. ncbi.nlm.nih.gov/database, accessed on Jan 13, 2009) was

(a)

	М	A	Ρ	Ρ	А	М	Е	Е	I	R	R	A	Q	R	A	Е	G	Ρ	А	Т	V	L	A	I	G	т	S	т	Ρ	Ρ	Ν	А
1	ATG	GCG	CCG	CCG	GCA	ATG	GAA	GAGA	ATC.	AGG	AGA	GCT	CAG	AGG	GCG	GAG	GGG	CCG	GCG	ACG	GTG	CTT	GCC.	ATC	GGA	ACC	TCC	ACG	CCG	CCG	AAC	ЗCТ
	L	Y	Q	А	D	Y	Ρ	D	Y	Y	F	R	I	Т	Κ	S	Е	Η	L	Т	Е	L	Κ	Е	K	F	Κ	R	М	С	D	K
97	CTG	TAT	CAG	GCG	GAC	TAT	CCG	GAT	TAC	TAC	TTC	AGG	ATC.	ACC.	AAG.	AGC	GAG	CAT	CTC	ACT	GAG	CTC	AAG	GAG	AAG	TTC	AAA	CGA	ATG	TGT	GAT.	AAA
	S	М	I	R	K	R	Y	М	Y	L	Т	Е	Е	Ι	L	K	Е	Ν	Ρ	Ν	I	С	A	F	М	А	Ρ	S	L	D	А	R
193	TCG	ATC	ATC	AGA	AAG	GCGC	TAC	ATG	TAC	TTA	ACA	GAA	.GAA	ATA	CTG	AAG	GAA	AAT	CCA	AAC	ATA	TGI	'GCA	TTC	ATG	GCG	CCA	TCA	CTA	GAC	GCA.	AGA
	Q	D	I	V	V	Т	Е	V	Ρ	K	L	A	K	Е	A	S	A	R	A	I	K	Е	W	G	Q	Ρ	K	S	R	Ι	Т	Н
289	CAA	AGAC	CATA	GTG	GTC	CACC	GAA	GTC	CCI	AAA	CTC	GCC	AAA	GAG	GCC	TCC	GCC	CGC	GCC	ATA	AAG	GAA	TGG	GGA	CAG	CCC	AAA	TCT	CGC	ATC.	ACT	CAT
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385	CTA	A'I'C	21'1'C	TGC	ACC	CACC	AGC	GGC	G'I'A	GAC	A'I'G	CCC	GGT	GCC	GAC	PAC	CAA	CTC	AC'I	CGC	CTC	CTC	GGC	CTC	CGC	CCA	TCC	GTC.	AA'I'	CGA	A'I'C.	A'I'G
4.0.1	L	Y Ama C	Q	Q	G	C	F.	A	G	G	T	A	Ц	R	L	A	К. 7777	D	L	A	E	N	N	A	G	A	R	V	L	V	V	C
481	CTT	TAC	CAP		GG 1	TGC	TTC	GCC	GGC	GGC	ACC	GCC	CTC	CGC	CIT	GCC	AAA	GAC	-CTC	GCC	GAG	AAC	AAC	GCC	GGC	GCG	ICGA	GIT	CTC	GTC	31.1.	TGT
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865	TTC	GAG	- CCG	- CTT	GGI	- ATT	CAC	GAC	TGG	AAT	TCG	- ATC	TTC	TGG	ATT	GCG	CAT	'CCG	GGC	GGT	CCG	GCG	- ATA	CTC	GAC	CÃA	.GTG	GAA	- ATT	AAG	CTT	GGA
	L	К	Е	Е	К	L	A	S	S	R	Ν	v	L	А	Е	Y	G	Ν	М	S	S	А	С	V	L	F	I	L	D	Е	М	R
961	CTT	AAC	GAA	GAG	AAG	GCTT	GCG	TCC.	AGC	AGA	AAC	GTG	CTT	GCG	GAG	TAT	GGC	AAT	ATG	TCC	AGC	GCG	TGT	GTG	CTT	TTC	ATA	CTT	GAT	GAA	ATG	AGG
	R	K	S	А	Е	A	G	Q	А	Т	Т	G	Е	G	L	Е	W	G	V	L	F	G	F	G	Ρ	G	L	Т	V	Е	Т	V
1057	CGC	GAAC	GTCC	GCA	GAG	GCG	GGG	CAG	GCI	ACC	ACC	GGC	GAG	GGG	TTG	GAG	TGG	GGA	GTA	CTG	TTC	GGA	TTT	GGC	CCG	GGG	CTT.	ACG	GTA	GAA	ACTO	ЭТТ
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1153	GTG	CTA	CGCI	AGCG	TTC	CGA	TTG	CTGG	TGC	GGT	GTG	Ą																				

(b)

DmCHS	(1)	MAPPAMEEIRRAQRAEGPATVLAIGTSTPPNALYQADYPDYYFRITKSEHLTELKEKFKRMCDKSMIRKRYMYLTEEILK
DsCHS	(1)	MAPPAMEEIRRAQRAEGPATVLAIGTSTPPNALYQADYPDYYFRITKCEHLTELKEKFKRMCEKSMIKKRYMYLTEEILK
DenCHS	(1)	-MAPAMEEIRRAQRAEGPAAVLAIGTSTPPNAVYQADYPDYYFRITNCEHLTDLKEKFKRMCEKSMIKKRYMYLTEEILK
PhalCHS	(1)	MAPPAMEEIRRAQRAEGPATVLAIGTSTPPNALYQADYPDYYFRITKSEHLTELKEKFKRMCDKSMIRKRYMYLTEEILK
DmCHS	(81)	ENPNICAFMAPSLDARQDIVVTEVPKLAKEASARAIKEWGQPKSRITHLIFCTTSGVDMPGADYQLTRLLGLRPSVIRIM
DsCHS	(81)	ENPNICAFMAPSLDARQDIVVTEVPKLAKEASTRAIKEWGQPKSRITHLIFCTTSGVDMPGADYQLTRLLGLRPSVIRIM
DenCHS	(80)	ENPNICAFMAPSLDARQDIVVAEVPKLAKEAAARAIKEWGHPKSRITHLIFCTTSGVDMPGADYQLTRLLGLRPSVIRFM
PhalCHS	(81)	ENPNICAFMAPSLDARQDIVVTEVPKLAKEASARAIKEWGQPKSRITHLIFCTTSGVDMPGADYQLTRLLGLRPSVIRIM
DmCHS	(161)	LYQQGCFAGGTALILAKDLAENNAGARVLVVCSEITAATFRGPSESHLDSLVGQALFGDGAAAIIVGSDPDLTTERPLFQ
DsCHS	(161)	LYQQGCFAGGTVLILAKDLAENNAGARVLVVCSEITAVTFRGPSESHLDSLVGQALFGDGAAAIIVGSDPDLTTERPLFQ
DenCHS	(160)	LYQQGCFAGGTVLILAKDLAENNAGARVLVVCSEITAVTFRGPSESHLDSLVGQALFGDGAAAIIVGSDPDLATERPLFQ
PhalCHS	(161)	LYQQGCFAGGTVLILAKDLAENNAGARVLVVCSEITAVTFRGPSESHLDSLVGQALFGDGAAAIIVGSDPDLTTERPLFQ
DmCHS	(241)	LVSASQTILPESEGAIDGHLREMGLTFHLLKDVPGLISKNIQKSLVEAFEPLGIHDWNSIFWIAHPGGPAILDQVEIKLG
DsCHS	(241)	LVSASQTILPESEGAIDGHLREMGLTFHLLKDVPGLISKNIQKSLVEAFKPLGIHDWNSIFWIAHPGGPAILDQVEVKLG
DenCHS	(240)	LVSASQTILPESEGAIDGHLREIGLTFHLLKDVPGLISKNIQKSLVEAFKPLGVLDWNSIFWIAHPGGPAILDQVETKLG
PhalCHS	(241)	LVSASQTILPESEGAIDGHLREMGLTFHLLKDVPGLISKNIQKSLVEAFKPLGIHDWNSIFWIAHPGGPAILDQVEIKLG
DmCHS	(321)	LKEEKLASSRNVLAEYGNMSSACVLFILDEMRRKSAEAGQATTGEGLEWGVLFGFGPGLTVETVVLRSVPIAGAV
DsCHS	(321)	LKAEKLAASRNVLAEYGNMSSACVLFILDEMRRRSAEAGQATTGEGLEWGALFGFGPGLTVETVVLRSVPIAGAV (99%)
DenCHS	(320)	LKSEKLAASRNVLAEYGNMSSACVLFILDEMRRRSAEAGQSTTGEGLEWGVLFGFGPGLTVEAVVLRSVPIGGTE (97%)
PhalCHS	(321)	LKAEKLASSRNVLAEYGNMSSACVLFILDEMRRRSAEAGQATTGEGLEWGVLFGFGPGLTVETVVLRSVPIAGAV (93%)

Fig. 2 Nucleotide and deduced amino acid sequence of *DmCHS* and alignment of the deduced amino acid sequences with other homologues. **a** Nucleotide and deduced amino acid sequence of *DmCHS*. The positions of nucleotides are given on the left. GenBank accession number for *DmCHS* is HQ412558. **b** The alignment of deduced amino

acid sequences of *DmCHS* with homologues from *Dendrobium* hybrid cultivar Sonia (*DsCHS*; CAM32716), *Dendrobium* hybrid (DenCHS; AAU93767), and *Phalaenopsis* hybrid (*PhalCHS*; AAY83389). *Shaded regions* show identical amino acids. *Box* indicates active site of chalcone synthases. The positions of amino acids are given on the *left*

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97	GTTA	GGGCA	ACAG	rgagi	AGAT(CCAA	CAAA		AAGZ	AAG	TGAZ	- 	TTTG	- CTG	GAT	_ СТС		 GCT	CCAZ	TGA		- 	AGCA	TTTGO	 \$AAA(GCA
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5//	ACIT	TGGTG	GTGG(JGIC		ATAA	CATC.	IGAA	ATGO	CAC	CAAC	JCAT(JTAC	ACT	GCA	TTA	TCA:		TTAC	AGG	AAA	GAT	GCCC.	ATTAC	TCC	ATT
	Ц ————————————————————————————————————	K Q	1 Q) F.	V	H I	L D	D	Ц.,	С	D A	A H	1	F.	L	F.	E	H	Pł	(A	N	G	R	Y I	С	S
673	'1''1'AA	AGCAA	ATTCA	AA'I''I''	TGTT	CATT	TGGA.	I'GAC	"I"I'A'	l'G'I'G	A'I'G(CTCA(CA'I''I	"1"1'C	CIT	1.1.1	GAG	CATC	CTAP	AGC	AAA'I	l'GG'I'.	AGA'I'.	ACA'I''I	"I'GC'.	I'C'I'
	S	Y D	S I	' I	Y	G 1	L A	E	М	L	K I	I R	Y	Р	Т	Y	A	I	PI	4 K	F	K	Е	I D	Р	D
769	TCCI	ATGAC	TCCA	CAAT'	TTAT	GGCT	TAGC	AGAA	ATG	CTGA	AGA	ACAG	ATAT	CCC	ACA	TAT	GCC	ATTC	CTCF	TAA	GTTI	[AAG	GAAA'	TTGAI	CCA	GAT
	I	к с	V S	5 F	S	S I	к к	L	М	Е	L C	5 F	K	Y	K	Y	Т	М	ΕH	E M	F	D	D	A I	K	Т
865	ATTA	AGTGI	GTAA	GCTT	CTCT	TCTA.	AGAA	GCTG	ATGO	GAGC	TTG	GTT	FAAG	TAC	AAA	TAC.	ACCI	ATGG	AGGI	GAT	GTTI	GAT	GATG	CAATC	AAG	ACC
	C	R E	K K	сL	I	ΡI	L N	Т	Е	Е	I	ΓL	A	A	Ε	K	F	Е	ΕV	/ К	Е	Q	I	A V	K	*
961	TGCA	GGGAG	AAGA	AGCT	TATA	CCAC	TCAA	CACT	GAG	JAAA	TAG	CTT2	AGCI	GCT	GAG.	AAA	TTT	GAGG	AAGT	TAA	AGAA	ACAG	ATTG	CTGTI	AAG	ΓGA
(h)																									
(D)																									
DmDI	7P	(1)	MENE	KKGP	WWW	GASG	VVGSW	T.VM	XT.T.O	KGVE	WRAT	יעפטי	TINT	KKVF	CPT.T	.DT.P	RSNI	21.15	TWKD	סם.דם	TEG	SEDE	VIRG	g		
BfDI	PD	(1)	MENE	VYCD		CASCI	VUCSW	T.VMI	ZT.T.O	KGYL		זחקדי	TINT:	FKVL	CDT.T.	ם.זת.	DOM	PLLC	TWKY		TEC	SEDE	VIRG	c c		
Cyml	סיזר	(1)	METE	PRCP		CASCI	VVCGW	T.VMI	ZT.T.O	KCVE			TNE	FKVR	CDT.T.		COM	T.T.C	TWKY	DLNE	ישמדע	TEDE	VTPC	c c		
Oper	- PFR	(1)	MCTE	NECT	VAVT	CA SCI	VUCSW	T.VMI	XT.T.O	KCVF			TINE.	FKVL		א.זם.	CGM	T.T.C	TWKY		TNE		VTRG			
one.	LDI'IC	(1)	MOID	11101	VAVIV	UCADO.	T VGDM		.уцп.б.	NOT 1			TINL.		ТЕПП	אננימו	0.0111	0000	1 1110		11112		V IICO	C		
DmDI	70	(01)	TOVE	ידע גענטי		OGVD	DENES	TOD		TTCT	TDC	אזאי	10110		2000	יא מיד	77 NT 7.7	PPU C	7775	יחשתי	wen	יישרו ד	VIDIN	м		
	FR. FD	(OI) (OI)	VCVE	TAVEL	DMNE	OGKD:	PENEV		ATNC	TTCT		TURC	10 V Q		2100		V IN V.	C C C C C C C C C C C C C C C C C C C			WSD	цре v т цеч	TURVE	1*1 1v1		
Grow	רת תיתר	(OI)	VGVF	TIVA1	DMNE	QGRD:	PENEV		TTCC	TTCT					2000	NOT NOT	V IN V.	PRUC		DENG	UGWGD	LULL N	TRVR	1*1 D/I		
Opar	JFR	(OI)	VGLF	TAVAL	PMNE	QSED:	PENEV	TVD	TTOG	ит ст	LLRS	JUDAC		RVII	7000	AGI		PRIIT	AIVI	DECC	WSD	TDET	TRVR	141		
one.	LDFR	(01)	VGIF	HVAI	PMINE	QSKD.	PENEV	INPI	ATING	IND G 1	LLRS		at v v	RVII	155	AGI	VIN V.	een.	ALVI	DESS	รพรม	LDFI	IRVR	141		
		(2.52)	marm						*** * *			ICOD	-		0. / T. P								TODA	**		
DmDI	r'R TD	(161)	TGWM	IYFLS	SKTLA	EKAA	WEFV	(DNH	THPT	TIII	DULTA DULTA	VGSF.	TTSE	MPP	SMIT	PALS	SLIT N. T.	GNDF	HYSI	LKQ.	LOFV	HLDL	LCDA	H		
BIDI	rR.	(161)	TGWM	IXEVS	K'I'LA	EKAA	WEFV	(ENA	THET	ALLI	5.I.P.A.	VGSF.	LINE	MPP	STL	L'ALS	SLIS	GNEA	HYSI	.LKQ#	AÕF.A	HLDD	LCDA	H		
Cyml	OFR	(161)	TGWM	IYFVS	SKTLA	EKAA	WEFVS	SDND	IHFI	TIII	PTLV	VGSF.	LISR	MPP	SLIT	FALS	SLIT	GNE	HYSI	LRQA	AQFV	HLDE	LCDA	H		
Onc	1DFR	(161)	TGMM	IXELS	skilta	EKAA	MEFAF	VDND	THET	ттт	5.I.PA	VGSF.	LISG	MPP	SMI	l'ALS	SPT.L	GNEF	HYSI	. I KQ#	AÕF.A	HLDD	LCDA	н		
		(0.000 C -					-					-									
DmDI	FR	(241)	IFLF	EHDR	ANGR	YICS	SYDSI	ΓIYG	LAEM	LKNI	RYPT.	YAIP	HKFK	EID	PDI	KCVS	FSS	KKTV	IELGE	KXKZ	Y T	MEEM	IFDDA	I		
BfDI	FR	(241)	IFVY	EHPE	ANGR	YICS	SHDSI	[IYD	LANM	LKNI	RYAT	YAIP	QKFK	EID	PNIF	KSVS	FSS	KKTV	IDLGE	KKK	Y T	IEEM	IFDDA	I		
CymI	OFR	(241)	IFLF	EHHK	ANGR	YICS	SHDSI	riys	LAKM	LKNI	RYAT	YDIP	LKFK	EID	PNIE	ESVS	SFSS	KKLI	DLGI	KXKZ	YKYT	MEEM	IFDDA	I		
Onc	idfr	(241)	IFLF	FEHPE	ANGR	YICS	SHDSI	ΓΙΥG	lakk	LKNI	RYVT.	YAIP	QKFK	DID	PDI	KSVS	SFSS	KKLN	IDLGE	KYKY	YT	MEEM	IFDDA	I		
			_					_																		
DmDl	FR	(319)	KTCF	SEKKT	IPLN	TEEI	VLAAI	EKFE	EVKE	QIA	VK	-														
BfDI	FR	(319)	KTCF	DKNL	MPLN	TEEL	VLAAI	EKYD	EVKE	QIA	VK	- (8	8응)													
CymI	OFR	(321)	KTCF	DKNL	IPLH	TEEM	VSANI	EKFD	EVKE	QIA	VK	- (84	4왕)													
Onc	iDFR	(319)	KSCF	DKNL	IPLN	TEKM	VSAAI	KFN	EIKE	KFCI	LVNN	E (8)	3응)													

Fig. 3 Nucleotide and deduced amino acid sequence of DmDFR and alignment of the deduced amino acid sequences with other homologues. a Nucleotide and deduced amino acid sequence of DmDFR. The positions of nucleotides are given on the left. GenBank accession number for DmDFR is HQ412559. b The alignment of deduced amino acid sequences of DmDFR with homologues from *Bromheadia*

used to detect similarities between cloned gene sequences and previously deposited sequences.

Gene Constructs

To construct the transient expression vector, the full-length *CHS*, *DFR*, and *F3'5'H* were placed between the *Cassava Vein Mosaic Virus* (CsVMV) promoter and the *Nos* terminator in the CsV vector (Verdaguer et al. 1996). Briefly, the full-length genes in pBluescript-T vector were digested with *XbaI* I and *Kpn* I, and then ligated into the same sites of the CsV vector to yield *pCsVMV::CHS*, *pCsVMV::DFR*, and *pCsVMV::F3'5'H*. To test promoter activity and bombardment efficiency, the *pCsVMV::GUS*

finlaysoniana (BfDFR; AAB62873), *Cymbidium* hybrid (*CymDFR;* AAC17843), and *Oncidium* Gower Ramsey (*OnciDFR;* AAY32602). *Shaded regions* show identical amino acids. *Box* indicates the NADP binding site. *Bold character* indicates the Asn of active site in the 135 residue. The positions of amino acids are shown on the *left*

construct was included. All constructs were confirmed by DNA sequencing.

Bombardment with Particle Inflow Gun

Near mature flowers were carefully excised from the plant using a razor and placed on the center of MS agar plate. DNA constructs were coated onto gold particles (1.5–3 μ m diameter; Sigma-Aldrich, USA) as described previously (Akashi et al. 2002). A biolistic helium device was used to microproject the DNA-coated gold particles into the perianths of *D. moniliforme* flower. Conditions for bombardment were as follows: reduced air pressure of –0.1 MPa, target distance of 12 cm, and helium pressure of 3.5 bar (a)

•	. /																			
	M S	I F	L I	T S	L	L L	C	L S	L	H L	L	L R	R	R I	ΙI	S	R L	Ρ	L P	P G
1 .	ATGTCTA	ATCTTC	CTCATC.	ACCTC	ACTCO	TCCT	CTGCCI	TTCT	CTCC.	ACCTC	CTCC	TCCGC	CGCC	GCCA	CATC	AGCC	GCTTA	CCCC	TCCCI	CCCGGC
	P P	N L	ΡI	I G	A	L F	P F	I G	Ρ	M P	Η	S G	L	A 1	L	A	R R	Y	G P	I M
97	CCCCCA	AACCTC	CCCATC	ATCGG	CGCCC	TCCC	CTTCAT	CGGC	CCCA	TGCCC	CACT	CCGGC	CTCG	CCCI	CCTT	GCCC	GCCGG	TAC	GCCC	CATCATO
	FL	K M	GΙ	RR	V	V V	7 A	s s	S	ТА	A	R T	F	LI	ΚТ	F	D S	Н	FS	DR
193	TTCCTC.	AAGATO	GGCATC	CGCCG	CGTC	STCGT	CGCCT	CTCC	TCCA	CCGCC	GCTC	GAACO	TTTC	TCAP	AACC	TTCG	ACTCC	CACI	TCTCC	CGACCGC
200	P S	G V		K E		S Y		y Q	N	M V	F	A D	Y magac	GI	P K	W	K L	L	K K	V S
289	CCCTCC	U I I	ATCICC	AAGGA	AATCA	AGCTA M C		JUCAG M N	AACA	TGGTC V D	TTCG	DE	TACG	T	CAAG	TGGA	AGCTC	T	V V	AGTCTCC
385	AGCCTC				. A GGCCI				יממרמ	TCCCC			A 1CCCT	יים. ידובידו		ב מתהע	2 F 7 7 7 7 7 7 7	ע יייייייייייייייייי		
505	D S	E K	P V	I CCAA	P	N I	. T. '	V C	۵۵۵۵۵ ۵	M A	N	V T	G	R	T A	M	S K	R	V F	H E
481	GATTCG	GAAAAG	CCGGTT	CTGCT	ACCA	AATTT	GTTGG	 TTTGT	GCCA	TGGCO	AATG	TGATI	GGGA	GGAI	CGCG	ATGA	GCAAA	AGAG	TGTT	CACGAG
	DG	ΕΕ	A K	ΕF	K	ΕM	1 I I	ΚE	L	l V	G	Q G	A	S I	N M	Е	D L	V	ΡA	I G
577	GACGGG	GAGGAG	GCGAAG	GAGTT	TAAG	GAGAT	GATTA	AGGGA	GCTG	TTGTO	GGGC	AGGG	GCTI	CGAA	TATG	GAGG	ATTTO	GTG	CCGGCC	GATCGGG
	W L	D P	M G	V R	K	K M	1 L	G L	Ν	R R	F	D R	М	V S	5 K	L	L V	Е	H A	Е Т
673	TGGTTG	GATCCO	ATGGGA	GTGAG	GAAGA	AAGAT	GCTGG	GATTG	AATC	GGAGG	TTTG	ATAGO	GATGG	TGAG	TAAG	TTGC	TGGTO	GAG	CACGCI	GAGACI
	A G	ER	Q G	N P	D	LI	ı D	ΓV	V	A S	Е	VК	G	ΕI) G	Е	G L	С	ΕD	N I
769	GCAGGG	GAGAGO	CAGGGG	AACCC	GGAT	CTGCT	GGATC	TGTT	GTGG	CTAGI	GAGG	TTAAZ	GGTG	SAGGA	TGGA	GAAG	GGCTI	FTGTO	JAAGA	TATAAT
0.05	K G	F I	S D			A G			5	A I	V		W	A I	M A	E	M L	К. 1777	N P	
865	AAGGGC	D A	O F	E T			GACGGA	ACACC	TCCG	D T.	IGTCA T.	TAGAC	9.1.9.9.6 G	CGA1	GGCA	GAAA	TGCTI T. D	V	ACCCCA	
961	CTCCGA	CGAGCO	CAAGAA	GAAAC	CGATO	CGCGT		3 10		GCCTT	UTGG	ACGAZ	ND.D.T.	ACAT	ACCA	AACC		- 77200	TCCA	AGCCATZ
201	C K	E A	L R	КН	P	P I	' P	L S	I	P H	Y	A S	E	P (C E	V	E G	Y	H I	P G
1057	TGCAAG	GAAGC	CTCCGA	AAGCA	CCCT	CCGAC	GCCGC	FCAGC	ATAC	CGCAC	TACG	CCTCC	GAGC	CCTG	CGAG	GTGG	AAGGO	CTAC	CACAT	CCCGGC
	Е Т	W L	L V	N I	W	A I	G	R D	P	d V	W	E N	P	L V	V F	D	P E	R	F L	Q G
1153	GAGACT	TGGCT	ACTCGTC	AACAT	ATGG	GCCAT	TGGGC	GGGAC	CCGG	ACGTO	TGGG	AGAAT	CCGI	TGGI	GTTC	GACC	CGGAG	GAGG	TTTCTC	GCAAGGG
	E M	A R	I D	P M	G	N D) F	ΕL	I	P F	G	A G	R	R I	I C	A	G K	L	A G	M V
1249	GAGATG	GCGAG	GATCGAT	CCGAT	GGGA	AATGA	TTTCG	AGCTC	ATAC	CGTTC	GGAG	CCGGG	CGGA	GGAI	TTGC	GCGG	GGAAG	GTTAC	GCGGGG	GATGGTO
1045	M V	Q Y	Y L	G '1	'L	VE	A	F D	W	S L	P	EG	V	GI	5 L 	D	ME	E	G P	G L
1345	ATGGTG	CAGTA.	TATTG	GGAAC D T	GITA(J'I'GCA	A TGCCT	I'TGA'I	TGGA	GTTTTC	iccgg	AAGGC	GTTG	iGGGA T T	GCTG	GACA	TGGAG	JGAAU	JGGCCC	-GGGGTTG
1441	GTGTTG	TCGAAG	A V GCTGTGC		GCGGI	rgatgi	GCGACG	CCGA	. ц заста		'GGCG	GCTTA	TGGC		ΓΤΤΑΑ					
1111	010110	0001110	0010100	.000111	.0000.	101110	0001100	CCOIR	50010		.0000	001111	1000	0110						
(h)																				
(D)																				
DmF3′	′5′H	(1) -	-MSIFLI	TSLLL	CLSLF	ILLLR	RRHISR	LPI	PPGP	PNLPI	TGAL	PFIGP	MPHS	TIATE	ARRY	GPTM	FT.KMG	TPDV	17177	
DgF3′	′5′H	(1) M		G 3 3 7 7	121237001											OT TIT	L DIG IG	T TOTO A	v v n	
PhalF			SISLFLA	GAALL	r r v i r	ILLLSI	PTR	- TRKI	PPGP	KGWPV	VGAL	PMLGN	MPHVA	ALANI	SRRY	GPIV	YLKLG	SRGM	VVA	
DenF3	F3′5′H	(1) -	-MSIFLI	GAAIL ATLFL	SLSLF	ILLLSI ILLLRI	PTR RFRRRR	-TRKI RILPI	LPPGP LPPGP	KGWPV LNFPI	VGAL: VGAL:	PMLGN PFIGS	MPHVA MPHSC	alani Glali	LSRRY LSRRY	GPIV GPIV	YLKLG FLKMG	SRGM	VVA VVA VVA	
	F3′5′H 3′5′H	(1) - (1) -	-MSIFLI -MSIFLI	GAAIL ATLFL TSLLL	SLSLI CLSLI	ILLLSI ILLLRI ILLLRI	PTR RFRRRR RRHRSR	- TRKI RILPI FPI	LPPGP LPPGP LPPGP	KGWPV LNFPI PNLPI	VGAL VGAL LGAL	PMLGN PFIGS PFIGP	MPHVA MPHS(MPHS(ALANI GLALI GLALI	LSRRY LSRRY LARRY	GPIV GPIM GPIM	YLKLG FLKMG FLKMG	SRGM IRQV IRRV	VVA VVA VVA	
Dmp2/	F3'5'H 3'5'H	(1) - (1) - (1)	-MSIFLI -MSIFLI	GAAIL ATLFL TSLLL	SLSLH CLSLH	ILLLSI ILLLRI ILLLRI	PTR RFRRRR RRHRSR	-TRKI RILPI FPI	LPPGP LPPGP LPPGP	KGWPV LNFPI PNLPI	VGAL VGAL LGAL	PMLGN PFIGS PFIGP	MPHV2 MPHSC MPHSC	ALANI GLALI GLALI	LSRRY LSRRY LARRY	GPIV GPIM GPIM	YLKLG FLKMG FLKMG	SRGM IRQV IRRV	VVA VVA VVA VVA	
DmF3'	F3'5'H 3'5'H '5'H	(1) - (1) - (77)	SISLFLA -MSIFLI -MSIFLI SSSTAAR	GAAIL ATLFL TSLLL TFLKT	FDSHF	ILLLSI ILLLRI ILLLRI 7SDRP:	PTR RFRRRR RRHRSR SGVISK	-TRKI RILPI FPI EISYN	JPPGP JPPGP JPPGP JGQNM	KGWPV LNFPI PNLPI VFADY	VGAL VGAL LGAL GPKW	PMLGN PFIGS PFIGP KLLRK	MPHV2 MPHS(MPHS(VSSLI	ALANI GLALI GLALI HLLGS	JSRRY JSRRY JARRY SKAMS	GPIV GPIM GPIM RWAG	YLKLG FLKMG FLKMG VRRDE	SRGM IRQV IRRV	VVA VVA VVA IQFL	
DmF3' DgF3' PhalF	F3'5'H 3'5'H '5'H '5'H 73'5'H	(1) = (1) = (77) (77) (77) (79)	SISLFLA -MSIFLI -MSIFLI SSSTAAR STPDSAR	GAAIL ATLFL TSLLL TFLKT AFLKT SFLKT	F F V I F SLSLH CLSLH FDSHF QDLNH	ILLLSI ILLLRI ILLLRI 7SDRP: 7SNRP:	PTR RFRRRR RRHRSR SGVISK IDAGAT	-TRKI RILPI FPI EISYN HIAYN	LPPGP LPPGP LPPGP IGQNM ISQDM	KGWPV LNFPI PNLPI VFADY VFADY	VGAL VGAL LGAL GPKW	PMLGN PFIGS PFIGP KLLRK KLLRK	MPHVA MPHSO MPHSO VSSLA LSSLA	ALANI GLALI GLALI HLLGS HMLGO	SRRY SRRY ARRY KARRY KAMS	GPIV GPIM GPIM GPIM GPIM GPIM	YLKLG FLKMG FLKMG VRRDE VRRDE	SRGM IRQV IRRV ALSM VGYM	VVA VVA VVA IQFL VKAI SHFL	
DmF3′ DgF3′ PhalF DenF3	F3'5'H 3'5'H '5'H '5'H F3'5'H 3'5'H	(1) - (1) - (77) (77) (79) (77)	SISLFLA -MSIFLI -MSIFLI SSSTAAR STPDSAR SSSSAAR SSATAAR	GAAIL ATLFL TSLLL TFLKT AFLKT SFLKT SFLKT	FDSHF QDLNF FDSHF	ILLLSI ILLLRI ILLLRI 7SDRP: 7SDRP 7SDRP1 7SDRP1	PTR RFRRRR RRHRSR SGVISK TDAGAT LDIISK SGVISK	-TRKI RILPI FPI EISYN HIAYN QVSYN EISYN	LPPGP LPPGP LPPGP IGQNM ISQDM IGQNM IGONM	KGWPV LNFPI PNLPI VFADY VFADY VFADY VFADY	VGAL VGAL LGAL GPKW GPRW GPKW	PMLGN PFIGS PFIGP KLLRK KLLRK KLLRK KLLRK	MPHVA MPHSO MPHSO VSSLI LSSLI VSNLI VSSLI	ALANI GLALI GLALI HLLGS HMLGC HLFGI HLLGS	SKAMS SKAMS SKAMS SKAIE SKAMS	GPIV GPIM GPIM GPIM GPIM GPIM GPIM GPIM GPIM	YLKLG FLKMG FLKMG VRRDE VRRDE VRRDE VRRDE	SRGM IRQV IRRV ALSM VGYM AFSM	VVA VVA VVA IQFL VKAI SHFL IOFL	
DmF3′ DgF3′ PhalF DenF3	F3'5'H 3'5'H '5'H '5'H F3'5'H 3'5'H	(1) - (1) - (77) (77) (79) (77)	SISLFLA -MSIFLI -MSIFLI SSSTAAR STPDSAR SSSSAAR SSATAAR	GAAIL ATLFL TSLLL TFLKT AFLKT SFLKT SFLKT	FDSHF CLSLF FDSHF QDLNF HDSRF FDSHF	ILLLSI ILLLRI ILLLRI FSDRPS FSDRPS FSDRPS	PTR RFRRRR RRHRSR SGVISK IDAGAT LDIISK SGVISK	-TRKI RILPI FPI EISYN HIAYN QVSYN EISYN	LPPGP LPPGP LPPGP IGQNM ISQDM IGQNM IGQNM	KGWPV LNFPI PNLPI VFADY VFADY VFADY VFADY	VGAL VGAL LGAL GPKW GPRW GPKW	PMLGN PFIGS PFIGP KLLRK KLLRK KLLRK KLLRK	MPHVA MPHSO MPHSO VSSLI LSSLI VSNLI VSSLI	ALANI GLALI HLLGS HMLGC HLFGI HLLGS	SKAMS SKAMS KAMS KAMS KAMS	GPIN GPIM GPIM RWAG' DWAV' RWAD RWAG	YLKLG FLKMG FLKMG VRRDE VRRDE VRRDE VRRDE	SRGM IRQV IRRV ALSM VGYM AFSM AFSM	VVA VVA VVA IQFL VKAI SHFL IQFL	
DmF3' DgF3' Phalf DenF3' DmF3'	F3'5'H 3'5'H '5'H '5'H F3'5'H 3'5'H '5'H	(1) - (1) - (77) (77) (79) (77) (157)	SISLFLA -MSIFLI -MSIFLI SSSTAAR STPDSAR SSSSAAR SSATAAR KKHSDS	GAAIL ATLFL TSLLL TFLKT AFLKT SFLKT SFLKT EKPVL	FDSHF CLSLF FDSHF QDLNF HDSRF FDSHF	ILLLS ILLLRI ILLLRI SDRP: SDRP: SDRP: SDRP: LVCAM	PTR RFRRRR RRHRSR SGVISK TDAGAT LDIISK SGVISK ANVIGR	-TRKI RILPI FPI EISYN HIAYN QVSYN EISYN IAMSP	LPPGP LPPGP LPPGP IGQNM ISQDM IGQNM IGQNM	KGWPV LNFPI PNLPI VFADY VFADY VFADY EDGEE	VGAL VGAL LGAL GPKW GPKW GPKW AKEF	PMLGN PFIGS PFIGP KLLRK KLLRK KLLRK KLLRK	MPHV2 MPHSC MPHSC VSSLI LSSLI VSNLI VSNLI VSSLI	ALANI GLALI GLALI HLLGS HMLGC HLFGH HLLGS GQGAS	LSRRY LSRRY LARRY SKAMS SKAIE SKAMS SKAMS	GPIN GPIN GPIM GPIM GPIM GPIM GPIM GPIM GPIM GPIM	YLKLG FLKMG FLKMG VRRDE VRRDE VRRDE VRRDE	SRGM IRQV IRRV ALSM VGYM AFSM AFSM	VVA VVA VVA VKAI SHFL IQFL RKKML	
DmF3' DgF3' Phalf DenF3 DmF3' DgF3'	F3'5'H 3'5'H '5'H '5'H F3'5'H 3'5'H '5'H '5'H	<pre>(1) - (1) - (77) (77) (79) (77) (157) (157)</pre>	-MSIFLI -MSIFLI SSSTAAR STPDSAR SSSSAAR SSATAAR KKHSDS YESSCA	GAAIL ATLFL TSLLL TFLKT AFLKT SFLKT SFLKT EKPVL GEAVH	FFVIF SLSLF CLSLF FDSHF HDSRF FDSHF LPNLI VPDMI	ILLLS ILLLRI ILLLRI ISDRP ISDRP ISDRP IVCAMI LVFAMI	PTR RFRRRR SGVISK IDAGAT LDIISK SGVISK ANVIGR ANMLGQ	-TRKI RILPI FPI EISYN HIAYN QVSYN EISYN IAMSF VILSF	.PPGP .PPGP .PPGP IGQNM ISQDM IGQNM IGQNM (RVFH &RVFV	KGWPV LNFPI PNLPI VFADY VFADY VFADY EDGEF TKGVE	VGAL: VGAL LGAL GPKW GPKW GPKW GPKW AKEF SNEF	PMLGN PFIGS PFIGP KLLRK KLLRK KLLRK KLLRK KEMIK KEMIK	MPHV2 MPHSC MPHSC VSSLI LSSLI VSNLI VSSLI ELLVC ELLVC	ALANI GLALI GLALI HLLGS HMLGC HLFGI HLLGS GQGAS SAGLI	SRRY SRRY ARRY KAMS KAIE KAMS KAMS KAMS	GPIN GPIN GPIM GPIM RWAG RWAG RWAG LVPA FIPS	YLKLG FLKMG FLKMG VRRDE VRRDE VRRDE VRRDE IGWLD IAWMD	SRGM IRQV IRRV ALSM VGYM AFSM AFSM PMGV LQGI	VVA VVA VVA VVA IQFL VKAI SHFL IQFL RKKML VRGMK	
DmF3' DgF3' Phalf DenF3 DmF3' DgF3' Phalf	F3'5'H 3'5'H '5'H '5'H F3'5'H 3'5'H '5'H '5'H '5'H ?3'5'H	<pre>(1) - (1) - (77) (77) (79) (77) (157) (157) (159)</pre>	-MSIFLI -MSIFLI SSSTAAR STPDSAR SSSAAR SSATAAR KKHSDS YESSCA KKQSDS	GAAIL ATLFL TSLLL TFLKT AFLKT SFLKT SFLKT EKPVL GEAVH KNPVL	F F V I F SLSLF CLSLF FDSHF QDLNF HDSRF FDSHF LPNLI VPDMI LSNLI	ILLLSI ILLLRI ILLLRI SSDRPS SSDRPS SSDRPS JVCAMI JVCAMI JVCSMI	PTR RFRRRR RRHRSR SGVISK IDAGAT LDIISK SGVISK ANVIGR ANMLGQ ANVIGR	-TRKI RILPI FPI EISYN HIAYN QVSYN EISYN IAMSP VILSF ISMSP	LPPGP LPPGP LPPGP IGQNM ISQDM IGQNM IGQNM IGQNM (RVFH RVFV (RVFD	KGWPV LNFPI PNLPI VFADY VFADY VFADY EDGEE TKGVE EEGKE	VGAL VGAL LGAL GPKW GPKW GPKW AKEF SNEF AKEF	PMLGN PFIGS PFIGP KLLRK KLLRK KLLRK KLLRK KEMIK KEMIK KEMIIK	MPHV2 MPHSC MPHSC VSSLH VSSLH VSSLH VSSLH ELLVC ELMTS ELLVC	ALANI GLALI GLALI HLLGS HMLGC HLFGI HLLGS GQGAS SAGLI GQGAS	SRRY SRRY ARRY KAMS KAMS KAMS KAMS SNMED NVGD SNIGD	GPIN GPIN GPIM RWAG' DWAV RWAG' RWAG' LVPA LVPA LVPA	YLKLG FLKMG FLKMG VRRDE VRRDE VRRDE VRRDE IGWLD IAWMD MRWLD	SRGM IRQV IRRV ALSM ALSM AFSM AFSM PMGV LQGI PQGA	VVA VVA VVA IQFL VKAI SHFL IQFL RKKML VRGMK RKKLL	
DmF3' DgF3' Phalf DenF3 DmF3' DgF3' Phalf DenF3	F3 · 5 · H 3 · 5 · H · 5 · H · 5 · H F3 · 5 · H 3 · 5 · H · 3 · 5 · H 3 · 5 · H	<pre>(1) - (1) - (77) (77) (79) (77) (157) (157) (159) (157)</pre>	-MSIFLI -MSIFLI SSSTAAR STPDSAR SSSSAAR SSATAAR KKHSDS YESSCA KKQSDS KKHSDT	GAAIL ATLFL TSLLL TFLKT AFLKT SFLKT SFLKT GEAVH KNPVL EKPVL	FPVIF SLSLF CLSLF QDLNF HDSRF FDSHF LPNLI LPNLI LSNLI LPNLI	ILLLS ILLLRI ILLLRI SSDRPS SSDRPS SDRPSDRPS SDRP	PTR RFRRRR RRHRSR SGVISK IDAGAT LDIISK SGVISK ANVIGR ANVIGR ANVIGR	-TRKI RILPI FPI EISYN HIAYN QVSYN EISYN EISYN IAMSF VILSF ISMSF IAMSF	LPPGP LPPGP LPPGP IGQNM ISQDM IGQNM IGNN IGNN IGNN IGNN IGNN IGNN IGNN IG	KGWPV LNFPI PNLPI VFADY VFADY VFADY VFADY EDGEE EEGEE EEGEE	VGAL VGAL LGAL GPKW GPKW GPKW GPKW SNEF AKEF AKEF	PMLGN PFIGS PFIGP KLLRK KLLRK KLLRK KLLRK KEMIK KEMIK KEIIK KEIIK	MPHV2 MPHSC WSSLI USSLI VSNLI VSSLI ELLVC ELLVC ELLVC ELLVC	ALANI GLALI GLALI HLLGS HMLGC HLFGI HLLGS GQGAS GQGAS GQGAS	SRRY SRRY ARRY KAMS KAMS KAMS KAMS KAMS SNMED SNIGD SNIGD	GPIN GPIN GPIM GPIM CWAG CWAG RWAG RWAG LVPA LVPA LVPA LVPA	YLKLG FLKMG FLKMG VRRDE VRRDE VRRDE IGWLD IGWLD IGWLD	SRGM IRQV IRRV ALSM VGYM AFSM AFSM LQGI PQGA PMGV	VVA VVA VVA IQFL VKAI IQFL IQFL RKKML VRGMK RKKLL KKRML	
DmF3' DgF3' PhalF DenF3 DmF3' DgF3' PhalF DenF3	F3 · 5 · H 3 · 5 · H · 5 · H · 5 · H F3 · 5 · H 3 · 5 · H · 5	(1) - (1) - (77) (77) (79) (77) (157) (157) (157) (159) (157)	-MS I FLI -MS I FLI -MS I FLI SSSTAAR STPDSAR SSSSAAR SSATAAR KKHSDS YESSCA KKQSDS KKHSDT	GAAIL ATLFL TSLLL TFLKT AFLKT SFLKT SFLKT EKPVL GEAVH KNPVL EKPVL	FPVIF SLSLF CLSLF FDSHF QDLNF HDSRF FDSHF FDSHF LPNLI LPNLI LPNLI	ILLLSI ILLLRI ILLLRI SSDRPS SSDRPS SSDRPS SSDRPS SDRPS	PTR RFRRRR RRHRSR SGVISK IDAGAT LDIISK SGVISK ANVIGR ANVIGR ANVIGR	-TRKI RILPI FPI EISYN HIAYN QVSYN EISYN IAMSP IAMSP IAMSP	LPPGP LPPGP JPPGP IGQNM ISQDM IGQNM IGQNM IGQNM IGQNM IGQNM IGQNM IGQNM IGQNM IGQNM IGQNM IGQNM	KGWPV LNFPI PNLPI VFADY VFADY VFADY EDGEE EEGEE EEGEE	VGAL VGAL LGAL GPKW GPKW GPKW GPKW SNEF AKEF AKEF	PMLGN PFIGS PFIGP KLLRK KLLRK KLLRK KLLRK KEMIK KEMIK KEIIK	MPHV2 MPHSC MPHSC VSSLI VSSLI VSSLI ELLVC ELLVC ELLVC	ALANI GLALI GLALI HLLGS HMLGC HLFGI HLLGS SQGAS SQGAS GQGAS	SRRY SRRY ARRY KAMS KAMS KAMS KAMS SKAMS SNMED SNMED SNIGD SNIGD	GPIN GPIN GPIM GPIM RWAG RWAG RWAG RWAG LVPA LVPA LVPA LVPS	YLKLG FLKMG FLKMG VRRDE VRRDE VRRDE IGWLD IGWLD IGWLD	SRGM IRQV IRRV ALSM VGYM AFSM AFSM PMGV PQGA PMGV	VVA VVA VVA IQFL VKAI SHFL IQFL RKKML VRGMK RKKLL KKRML	
DmF3' DgF3' PhalF DenF3' DgF3' PhalF DenF3 DmF3'	F3 · 5 · H 3 · 5 · H · 5 · H F3 · 5 · H 3 · 5 · H 3 · 5 · H · 5 · H F3 · 5 · H 3 · 5 · H 3 · 5 · H 3 · 5 · H	<pre>(1) - (1) - (77) (77) (79) (77) (157) (157) (157) (159) (157) (237)</pre>	-MSIFLA -MSIFLI -MSIFLI SSSTAAR STPDSAR SSSSAAR SSATAAR KKHSDS YESSCA KKQSDS KKHSDT GLNRRF	GAAIL ATLFL TSLLL TFLKT AFLKT SFLKT SFLKT EKPVL EKPVL EKPVL DRMVS	FDSHF CLSLF FDSHF QDLNF HDSRF FDSHF LPNLI LPNLI LSNLI LPNLI	ILLLSI ILLLRI ILLLRI SSDRPS SSDRPS SSDRPS SSDRPS SVCAMI SVCAMI SVCAMI SVCAMI	PTR RFFRRR RRHRSR SGVISK IDAGAT LDIISK SGVISK ANVIGR ANVIGR ANVIGR ANVIGR	-TRKI RILPI FPI EISYN HIAYN QVSYN EISYN IAMSF VILSF ISMSF IAMSF NPDLI	LPPGP LPPGP LPPGP IGQNM IGQNM IGQNM IGQNM (RVFH RVFV (RVFD (RVFD LDLVV	KGWPV LNFPI PNLPI VFADY VFADY VFADY VFADY EDGEE EEGEE EEGEE ASEVK	VGAL VGAL LGAL GPKW GPKW GPKW AKEF SNEF AKEF AKEF	PMLGN PFIGS PFIGP KLLRK KLLRK KLLRK KLLRK KEMIK KEMIK KEMIK EGLCE	MPHVZ MPHSC MPHSC VSSLI VSSLI VSSLI ELLVC ELLVC ELLVC DNIKC	ALANI GLALI GLALI HLLGS HMLGC HLFGI HLLGS SAGLI GQGAS GQGAS GFISI	SRRY SRRY ARRY KAMS KAMS KAMS KAMS SKAMS SNMED SNIGD SNIGD SNIGD	GPIN GPIN GPIM GPIM RWAG DWAV RWAG RWAG RWAG LVPA LVPA LVPA LVPA LVPA	YLKLG FLKMG FLKMG VRRDE VRRDE VRRDE IGWLD IGWLD IGWLD IGWLD	SRGM IRQV IRRV ALSM VGYM AFSM AFSM PMGV LQGI PQGA PMGV EWAM	VVA VVA VVA IQFL VKAI SHFL IQFL RKKML VRGMK RKKLL KKRML AEMLK	
DmF3' DgF3' PhalF DenF3' DgF3' PhalF DenF3 DmF3' DgF3' PhalF	F3 (5 'H 3 (5 'H 3 (5 'H 5 'H F3 (5 'H 3 (5 'H 3 (5 'H 3 (5 'H 3 (5 'H 3 (5 'H 5 'H 5 'H 5 'H 5 'H	<pre>(1) - (1) - (77) (77) (79) (77) (157) (157) (157) (157) (157) (237) (237)</pre>	-MSIFLA -MSIFLI -MSIFLI SSSTAAR STPDSAR SSSSAAR SSATAAR KKHSDS YESSC2 KKQSDS KKHSDI GLNRRF RLHNFF	GAALL ATLFL TSLLL TFLKT AFLKT SFLKT SFLKT GEAVH KNPVL EKPVL DRMVS DALLD VRMIS	FPVIF SLSLF CLSLF FDSHF QDLNF HDSRF FDSHF LPNLI LPNLI LSNLI LSNLI LSNLI LSNLI KLLVF KILRF KFLAF	ILLLSI ILLLRI ILLLRI ILLLRI SSDRPS SSDRPS SSDRPS SVCAMA SVCAMA SVCAMA SUCAMA SUCAMA SUCAMA SUCAMA SUCAMA SUCAMA SUCAMA	PTR RFRRRR RRHRSR SGVISK IDAGAT LDIISK SGVISK ANVIGR ANVIGR ANVIGR ANVIGR AGERQG RSERKG	-TRKI RILPI FPI EISYN HIAYN QVSYN EISYN IAMSF ISMSF IAMSF NPDLI KPDLI	LPPGP LPPGP LPPGP IGQNM IGQNM IGQNM IGQNM (RVFH (RVFD (RVFD (RVFD LDLVV /DVLM	KGWPV LNFPI PNLPI VFADY VFADY VFADY VFADY EDGEE EEGEE EEGEE ASEVK ADKTA	VGAL VGAL LGAL GPKW GPKW GPKW SNEF SNEF AKEF AKEF GEDG KSEQ GDDG	PMLGN PFIGS PFIGP KLLRK KLLRK KLLRK KLLRK KEMIK KEMIK KEMIK EGLCE ERLTD EGLSE	MPHVZ MPHSC MPHSC VSSLI USSLI VSSLI ELLVC ELLVC ELLVC DNIKC TNIKZ	ALANI GLALI GLALI HLLGS HMLGC HLFGI HLLGS GQGAS GQGAS GQGAS GGFISI ALLLN CFISI	SRRY SRRY ARRY KARS KAMS KAMS KAMS KAMS KAMS KAMS KAMS KAM	GPIV GPIM GPIM GPIM RWAG RWAD RWAG LVPA LVPA LVPA LVPA LVPA STDT GTDT	VRRDE VRRDE VRRDE VRRDE VRRDE IGWLD IGWLD IGWLD SAIVI SSSTI SSSTI	SRGM IRQV IRRV ALSM AFSM AFSM PMGV PQGA PMGV EWAM EWAM	VVA VVA VVA VVA VKAI SHFL IQFL RKKML VRGMK RKKLL KKRML AEMLK TEMIK AEMLK	
DmF3' DgF3' PhalF DenF3' DgF3' PhalF DenF3 DmF3' DgF3' PhalF DenF3	F3 (5 'H 3 (5 'H 5 'H 5 'H F3 (5 'H 3 (5 'H 5 'H 5 'H 7 5 'H 7 3 (5 'H	<pre>(1) - (1) - (77) (77) (79) (77) (157) (157) (157) (157) (237) (237) (237)</pre>	-MSIFLI -MSIFLI -MSIFLI SSSTAAR STDDSAR SSSSAAR SSATAAR KKHSDS VESSCA KKQSDS KKHSDT GLNRRF RLHNKF GLNRRF GLNRRF	GAALL ATLFL TSLLL TFLKT AFLKT SFLKT SFLKT GEAVH KNPVL EKPVL DRMVS DALLD VRMIS DRMVS	FPVIF SLSLF CLSLF FDSHF QDLNF HDSRF FDSHF LPNLI LPNLI LPNLI LSNLI LPNLI KLLVF KILRF KFLAF	ILLLSI ILLLSI ILLLRI ILLLRI SSDRPS SSDRPS SDRPS	PTR RFRRRR RRHRSR SGVISK IDAGAT LDIISK SGVISK ANVIGR ANVIGR ANVIGR ANVIGR ANVIGR AGERQG RSERKG RGEREG AGERCG	-TRKI RILPI FPI EISYN HIAYN QVSYN EISYN IAMSF IAMSF IAMSF NPDLI KPDLI NPDLI NPDLI	LPPGP LPPGP LPPGP IGQNM IGQNM IGQNM IGQNM IGQNM IGQNM IGQNM IGQNM IGQNM IGQNM IGQNM LDLVV LDLVV LDLVV DLVV	KGWPV LNFPI PNLPI VFADY VFADY VFADY EDGEE TKGVE EEGEE ASEVK DNRDN ADKIA GSELI	VGAL VGAL LGAL GPKW GPKW GPKW GPKW SNEF AKEF AKEF GEDG GEDG GEDG	PMLGN PFIGS PFIGP KLLRK KLLRK KLLRK KEMIK KEMIK KEMIK EGLCE EGLCE EGLCE EGLCE	MPHVZ MPHSC MPHSC VSSLI VSSLI VSSLI ELLVC ELLVC ELLVC DNIKC ENIKC	ALANI GLALI GLALI GLALI HLLGS HLLGS GQGAS GQGAS GQGAS GGA	SRRY SRRY ARRY KARS KAMS KAMS KAMS KAMS KAMS KAMS KAMS KAM	GPIV GPIM GPIM GPIM RWAG RWAG RWAG RWAG LVPA LVPA LVPA LVPA LVPA GTDT GTDT GTDT	VRRDE VRRDE VRRDE VRRDE VRRDE IGWLD IGWLD IGWLD SAIVI SAIVI SAIVI	SRGM IRQV IRRV ALSM AFSM AFSM DQGI PQGA PMGV EWAM EWAL EWAM	VVA VVA VVA VVA VVA IQFL VKAI SHFL IQFL RKKML VRGMK RKKLL KKRML AEMLK AEMLK AEMLK	
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PhalF3'5'H	(479)	GPGLVLPKAVPLLVTARPRLPAAAYGVV	(84%)
DenF3′5′H	(477)	GPGLVLPKAVPLSVMARPRLAPAAYGLL	(95%)

Fig. 4 Nucleotide and deduced amino acid sequence of DmF3'5'H and alignment of the deduced amino acid sequences with other homologues. a Nucleotide and deduced amino acid sequence of DmF3'5'H. The positions of nucleotides are given on the *left*. GenBank accession number for DmF3'5'H is HQ412560. b The alignment of deduced amino acid sequences of DmF3'5'H with homologues from *Delphinium*

(50 psi). The bombarded floral organs were then incubated on MS agar medium in 12-h light/12-h dark cycle at 25°C. Three to five days after bombardment, the development of grandiflorum (DgF3'5'H; AAX51796), Phalaenopsis hybrid (PhalF3'5' H; AAZ79451), and Dendrobium hybrid (DenF3'5'H; ABI95365). Shaded regions show identical amino acids. Box indicates the Cytochrome P450 cysteine heme-iron ligand signature. The positions of amino acids are shown on the left

pigmentation was analyzed in the perianth organ. GUS activity was measured by histochemical staining as described (Koo et al. 2007). Pigmentation color was analyzed and



Fig. 5 Expression analysis of three anthocyanin biosynthetic genes in *Delphinium moniliforme* floral organs. **a** Semi-quantitative RT-PCR analysis. First-stranded cDNA was synthesized using total RNA extracted from petals (*Petal*), sepals (*Sepal*), lip (*Lip*), and column including the base (*BC*). *Actin* (*DmActin*) gene was included in the experiment as an internal control. The PCR amplified products were analyzed by 1.5% agarose gel electrophoresis. **b** Quantitative RT-PCR analysis. One twentieth of first-strand cDNA mixture was used as template. Relative quantity of each gene expression is represented by to one fourth level of *actin* mRNA. *Error bars* indicate SD (n=3)

presented as value according to PCCS 199a Harmonic Color Charts (COJI, Japan).

Results and Discussion

Isolation of Three Anthocyanin Biosynthetic Genes from *D. moniliforme*

D. moniliforme is a species of genus *Dendrobium* found in the southern areas of Korea. Like other orchids, the *D. moniliforme* flower consists of a reproductive column and three types of perianth organs including three outer tepals (called sepals), two inner tepals (petal) and a labellum (lip) (Fig. 1a–c). Interestingly, the base of the column, which is fused with the column, bears vivid reddish purple spots compared with white perianths. Color value is v23 according to the PCCS Harmonic Color Charts. This may

suggest that white perianths of *D. moniliforme* are due to transcriptional control of spatially regulated pigment bio-synthetic genes and/or their regulatory genes.

To test this hypothesis, we initially attempted to isolate the genes that are involved in the anthocyanin biosynthetic pathway since anthocyanins have been reported to mainly contribute diverse flower colors in *Dendrobium* (Arditti and Fisch 1977). In particular, sense and antisense expression of *CHS*, *DFR*, and *F3'5'H* (Fig. 1d) have been the most exploited to modulate flower color and/or intensity (van der Krol et al. 1990; Courtney-Gutterson et al. 1994; Jorgensen et al. 1996; Tanaka et al. 1998). To isolate these genes in *D. moniliforme*, degenerate primers were designed based on conserved regions of the previously reported sequences in GenBank (see "Materials and Methods").

Using RT-PCR with degenerate primers, a full-length cDNA encoding CHS was cloned by amplifying a 1,188 bp sequence from the base of the column and designated *DmCHS* (Fig. 2a). BLASTP searches with the deduced amino acid sequences of *DmCHS* showed 99%, 97% and 93% identity with those of *Dendrobium* hybrid cultivar Sonia (accession no. CAM32716; Pitakdantham et al. 2010), *Dendrobium* hybrid (accession no. AAU93767; Mudalige-Jayawickrama et al. 2005), and *Phalaenopsis* hybrid (accession no. AAY83389; Han et al. 2006), respectively (Fig. 2b). An essential catalytic site for CHS and possible binding site for the 4-coumaryl-CoA group was found in the sequence as a consensus pattern of "R-[LIVMFYS]-x-[LIVM]-x-[QHG]-x-G-C-[FYNA]-[GAPV]-G-[GAC]-[STAVK]-x-[LIVMF]-[RAL]" (Lanz et al. 1991).

In the same manner, a full-length cDNA encoding DFR was isolated from the base of the column and designated as DmDFR (Fig. 3a). The length of DmDFR was 1,056 bp encoding for a protein of 147 amino acids. The protein sequence of DmDFR showed 88%, 84%, and 83% identity to those from Bromheadia finlaysoniana (AAB62873), Cymbidium hybrid (AAC17843), and Oncidium Gower Ramsey (AAY32602; Mudalige-Jayawickrama and Kuehnle 2006) (Fig. 3b). In addition, the consensus sequence of the NADPH-binding domain, found in the DFR superfamily (Baker and Blasco 1992), was conserved in the N-terminal region of DmDFR. Although DFR catalyzes the conversion of DHK, DHQ, and DHM to leucoanthocyanidins (Fig. 1a), certain types of DFR in some plants lack the ability to reduce DHK. For example, Petunia DFR was reported to lack the ability to convert DHK to leucopelargonidin (Forkmann and Ruhnau 1987) due to a substitution of Asn to Asp at the 134 residue of the active site (Beld et al. 1989; Johnson et al. 2001). In DmDFR, amino acid residue 135 (corresponding 134 residue of petunia or Gerbera) is an Asn as observed in other Dendrobium species (Mudalige-Jayawickrama et al. 2005), implying that DmDFR contains functionally active Asn residue in 135 position.

The F3'5'H clone, designated as DmF3'5'H, is 1,512 bp in length encoded a protein of 504 amino acid residues (Fig. 4a). F3'5'H catalyzes the hydroxylation of DHK at both 3' and 5' positions of the B-ring that leads to the formation of DHM and subsequently to the production of delphinidin-based pigments (purple to violet) as shown Fig. 1a. Sequence comparison revealed that deduced amino acid sequence of DmF3'5'H shared 56%, 84%, and 95% identity compared with those from Delphinium grandiflorum (AAX51796), Phalaenopsis hybrid (AAZ79451), and Dendrobium hybrid (ABI95365) (Fig. 4b). F3'5'H belongs to the cytochrome P450 superfamily (Holton et al. 1993). The consensus sequence of cytochrome P450 signature controlling flower color was well conserved in DmF3'5'H. Taken together, these data strongly suggest that the three isolated cDNA clones may be typical types of anthocyanin biosynthetic genes.

Expression Analysis of Anthocyanin Biosynthetic Genes in Floral Organs

Colorless perianths of *D. moniliforme* flower could be explained by several reasons. These may include the spatiotemporal regulation of anthocyanin biosynthetic genes and/ or their regulatory genes, or mutational effects on the structural genes encoding enzymes for anthocyanin biosynthesis. However, we focused on the first possibility since reddish purple pigmentation spots were locally accumulated in the base of the column of the flower (Fig. 1c, d).

To determine the expression patterns of three anthocyanin biosynthetic genes in floral organs in detail, semi-quantitative RT-PCR was performed using total RNA isolated from sepals, petals, lip and column including the base. CHS controls first step in anthocyanin biosynthesis, thus we assume that its expression is lowered or absent in the perianths of the D. moniliforme flower. In contrast to our expectation, the CHS transcript level was slightly higher or not significantly different in sepals, lip, and petal compared with the base of the column (Fig. 5a, b), respectively. In addition, the expression of DFR exhibited no significant differences among floral organs. However, the F3'5'H transcripts level in the base of the column was significantly higher than that of other floral organs. Quantitative RT-PCR analysis also confirmed that F3'5'H expression is approximately 3-fold higher in the base of the column compared with other organs (Fig. 5b). Therefore, the result suggested that colorless of the perianth organs was attributed to a lowered expression of F3'5'H expression. This, in turn, may suggest that the F3'5'Hexpression is controlled by spatially regulated transcriptional factor(s) in D. moniliforme flower.

Development of Pigmentation by Transient Expression of DmF3'5'H

As shown in RT-PCR analysis (Fig. 5), pigment accumulation in the base of column may be due to the preferential expression of DmF3'5'H. Thus, we expected that expression of this gene would complete the anthocyanin pathway and produce anthocyanin compounds to display the similar pigment in perianth. To test this, the full-length DmF3'5'Has well as DmDFR and DmCHS genes were individually cloned into CsV999 vector (Verdaguer et al. 1996) that contained the constitutive CsVMV promoter and NOS terminator. Since the activity of CsVMV promoter was



Fig. 6 Development of reddish purple spots in white perianth of *D.* moniliforme flower by transient expression of DmF3'5'H gene. The constructs including DmCHS (b), DmDFR (c), and DmF3'5'H (d) were bombarded into perianth organs. Bacterial *GUS* gene was included as a control (a). Transient expression of bombarded genes

were driven using the constitutive CsVMV promoter. After 3 to 5 days of incubation, GUS activity or pigmentation was examined under the stereomicroscope. Representative images from two independent experiments were shown. *Scale bar* indicate 1 mm

not tested in Dendrobium flower before, pCsVMV::GUS construct was also included to validate promoter activity and bombardment efficiency. These constructs were then bombarded into white perianths (Fig. 6). After incubation on MS agar for three to five days, distinct GUS staining spots were observed in the perianths bombarded with *pCsVMV::GUS* construct, suggesting that *CsVMV* promoter was active in perianths of D. moniliforme flower. Under the same condition, bombardment with pCsVMV::DmF3'5'H resulted in development of reddish purple pigment spots in perianth organs, corresponding to v23 or v24 value of PCCS 199a color charts. However, perianth organs bombarded with pCsVMV::DmCHS or pCsVMV::DmDFR did not show noticeable signs of pigmentation during the assay period. These results indicated that expression of DmF3'5'Hcould completely rescued the absence of anthocyanin biosynthesis in perianth organs.

In this study, we demonstrated that transcriptional activation of DmF3'5'H gene is critical for coloration in the perianths of *D. moniliforme* flower. Therefore, it is interesting to determine which regulatory component(s) is responsible for spatial expression of DmF3'5'H gene in *D. moniliforme* flower.

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