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ORIGINAL RESEARCH

Identification of Differentially Expressed Genes in Flower Buds of *Calanthe discolor* and *C. sieboldii*

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Abstract The genus *Calanthe* includes species of terrestrial orchids that produce attractive flowers with diverse floral traits. Breeding programs have been established to improve the horticultural value of various *Calanthe* species, but studies to identify the genetic components contributing to the key phenotypic characteristics have not been undertaken. To understand the molecular mechanisms underlying floral development associated with floral morphology, color, and fragrance production, the flower buds of two typical Korean *Calanthe* species, *C. discolor* and *C. sieboldii*, were subjected to gene expression analysis by differential display RT-PCR (DDRT-PCR). A total of 66 non-redundant differentially expressed genes (DEGs) were isolated and sequenced. Of these, 26 and 40 DEGs were found to be highly expressed in *C. discolor* and *C. sieboldii*, respectively. Moreover, the expression patterns of a subset of genes presumably implicated in signal transduction, metabolic pathways, and hormonal signaling differed between the two species. The data presented here may improve our understanding of the mechanisms underlying floral development and contribute to advances in orchid biotechnology.

Keywords Differentially expressed gene (DEG) · Floral gene · DDRT-PCR

Abbreviations

DEG	Differentially expressed gene
RT-PCR	Reverse transcription-polymerase chain reaction
ACP	Annealing control primer

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Introduction

The genus *Calanthe*, a member of the Orchidaceae, is widely distributed in tropical and subtropical regions, including Africa, the Pacific Islands, Southeast Asia, China, Japan, and Korea (Gale and Drinkell 2007). Although this genus comprises about 171 species worldwide, only four species (*C. discolor* Lindl, *C. sieboldii* Ohwi, *C. reflexa* Maximowicz, and *C. aristulifera* Hayata) and a natural hybrid between *C. discolor* and *C. sieboldii* (*C. bicolor* Lindl) are reported to occur naturally in Korea (Lee and Kwack 1983; Kim and Kim 1989; Hyun et al. 1999a, b). Depending on taxonomic and nomenclatural aspects, *C. sieboldii* is often classified into *C. discolor* f. *sieboldii* (Dence) Ohwi (Iwatsuki 1995; Kim et al. 2008; Korea Plant

Name Index 2007). Among them, the most popular and captivating species are *C. discolor* and *C. sieboldii*. While these two species do not exhibit significant morphological differences during vegetative growth, the flowers of *C. sieboldii* are slightly larger and exude a stronger fragrance than those of *C. discolor*. Furthermore, the flowers of *C. sieboldii* are uniformly yellow, whereas those of *C. discolor* bear bicolored flowers with brownish outer tepals (often called sepals) and lateral inner tepals (petals), and a white median inner tepal (lip).

Flower color is one of the most important traits in ornamental plants. It is well established that flavonoids, carotenoids, and betalains are largely responsible for the broad range of flower colors, from yellow to red, violet, and blue. Carotenoids mainly contribute to the production of yellow and orange pigments, such as those present in sunflowers and tomatoes (Bartley and Scolnik 1989). Betalains are yellow to red nitrogenous compounds distributed only in *Caryophyllales* (Stafford 1994). Anthocyanins, a class of flavonoids, are the major floral pigments in higher plants and accumulate in vacuoles (Goto and Kondo 1991). The biosynthetic pathways underlying the production of these pigments are generally conserved among plant species, and the genes that encode the enzymes involved in pigment biosynthesis have mostly been isolated (Holton and Cornish 1995; reviewed in Tanaka and Ohmiya 2008). Flower coloration is also thought to be specified by temporal and spatial expression patterns of the regulatory genes (Ludwig and Wessler 1990; Holton and Cornish 1995), suggesting that detailed studies of the transcriptional control of biosynthetic genes may lead to the identification of novel regulatory components.

Flowers of the *Calanthe* vary greatly in terms of color, shape, fragrance, and longevity. Therefore, conventional breeding programs have been conducted to improve the horticultural traits and genetic diversity within a species of *Calanthe* or a range of closely related *Calanthe* species. Recently, a molecular linkage map and quantitative trait loci (QTL) analysis identified four QTLs that are closely associated with flower and lip color expression in *C. discolor*, *C. sieboldii*, and variants (Cho et al. 2009). However, the molecular mechanisms underlying key biological processes and the identification of the critical components contributing to phenotypic variation remain largely unknown. Most molecular studies of *Calanthe* have been limited to taxonomic classification (Hyun et al. 1999a, b; Gale and Drinkell 2007; Silveira et al. 2008). Indeed, all DNA sequences deposited for this genus in Genbank thus far belong to molecular markers with applications in phylogenic studies. To identify the genes that play key roles in floral development and encode proteins that participate in the underlying metabolic pathways, we analyzed the differentially expressed genes (DEGs) that contribute to the distinct characteristics of flowers from the two *Calanthe* species, *C. discolor* and *C. sieboldii*. Based on differential display RT-PCR (DDRT-PCR) and gene-specific RT-PCR, a subset of key regulatory genes, presumably involved in a signaling pathway, secondary metabolism, and hormone pathways, were identified as being differentially expressed in the floral organs of the two species. Our data presented here may be a useful resource for scientists focusing on orchid floral development and biotechnology.

Materials and Methods

Plant Materials

Two *Calanthe* species, *C. discolor* Lindl and *C. sieboldii* Ohwi, were collected from Jeju Island in Korea and cultivated for over 5 years in the greenhouse facility of Chonbuk National University. Young flower buds were harvested just before flowering in late April from five individual plants of each species, and the buds from each species were combined and kept at −70°C until used. Total RNA was extracted from the two sets of combined samples.

Total RNA Extraction, DDRT-PCR, and Semiquantitative RT-PCR

Total RNA was extracted using Tri reagent (MRC, USA) and then treated with RQ1-DNase I (Promega, Germany) to remove DNA contamination, according to the manufacturers' instructions. Samples were then analyzed by DDRT-PCR technology using the Gene Fishing Kit (Seegene, Inc., Korea), according to the manufacturer's instruction. Briefly, 3 μg of total RNA was reverse transcribed using the dT-ACP1 primer and M-MLV reverse transcriptase. Then, 1/30 of the first-stranded cDNA samples was used as a template for PCR in combination with 120 arbitrary ACPs and the dT-ACP2 primer set. PCR conditions were as follows: 1 cycle of 94°C for 5 min, 50°C for 3 min, and 72°C for 1 min; 40 cycles of 94°C for 40 s, 65°C for 40 s, and 72°C for 40 s; and a final extension period of 72°C for 5 min. The resulting PCR products were separated and visualized on a 1.2% agarose gel. After ethidium bromide staining, the differentially expressed products were excised from the gel and cloned into a pBluescript-T vector that is modified for cloning of PCR products (kind gift from Dr. Hwang Inhwan, POSTECH, Korea) for DNA sequencing.

Semiquantitative RT-PCR was performed to confirm the differential expression of the identified cDNA clones using the gene-specific primers listed in Table 1. Total RNA (3 μg) was used for cDNA synthesis, and 1/10 of the resulting volume of cDNA was used as template for the

Table 1 Sequences of the gene-specific primers used for RT-PCR analysis

cDNA clone	Primers	Amplicon (bp)
Cs004 (4-coumarate-coA ligase)	5′-GTGGGGCAGATACCTATGGC-3′ 5′-TGCCATACAGACCAGGGAGAGG-3′	323
Cs006 (annexin P35)	5′-AGTGGCGCTGGTAAGCTCCT-3′ 5′- TTGCTGCATCCAAAGCCTTGCT-3′	437
Cs012 (SAMS)	5′-ACGTACGGCGGT TGGGGAGC-3 5′-GATTGCCCTTTGTATCCCAACGG-3′	420
Cs015 (IAA1)	5′- TTTGACCGCGGAGTCCTCCG -3′ 5′- GATGGTGCTGCAGCTCTAGCC -3′	63
Cs019 (GTPase activating protein)	5′-CCAGATCCTATTCCGCCCCT-3′ 5′-GGGGCATGCATTCAAGCTCC-3′	298
Cs020 (myo-inositol-1-phosphate synthase)	5′-TCAAGGCTGAGGGAGAGGGA-3′ 5′-AGAACCGGGCCTTGAGAAGG-3′	301
Cs021 (fibrillin-like protein)	5′-ACGAAGCCCCAAACGTGTGC-3′ 5′-AACTCAAGAGAGCGCTGCCT-3′	332
Cs034 (GTPase SAR1)	5′-TGCCTCGCTAGGGCTGTGGC-3′ 5′-GGCCCAGGTGGTAGCGGAGC-3′	410
Cs039 (low temperature responsive protein)	5′- CATCCTTCTCCCGCCGCTTG-3′ 5′- AGGTCGAGGAACAAGCATCGTCT-3′	207
Cd005 (unknown)	5′- TATCCTCCTCTGCAAGAACG-3′ 5′- CAGGTACCGACCATACTAGC-3′	460
Cd006 (ATP citrate lyase)	5′-GACAAGAGAGTTCAGCTACCCCA-3′ 5′- ATCTTCCCATGGGTGGCGGT-3′	321
Cd008 (calmodulin)	5′-GCCGTACACGGAAGAGCAGC-3′ 5′-CAAAGCGGAGACATGTGGGAC-3′	346
Cd024 (SAMS)	5′-CCTTCTCCGGCAAGGATCCGACAAAG-3′ 5′-TTCATGACCATATCATCGGCTACACAC-3′	433
Cd025 (flavonoid 3′-hydroxylase)	5′- GGGACGTGCAGGATCTCCCA-3′ 5′- CCCATCGGGCAGTCGCCAGC-3′	374
Cal_Actin	5′- ATGTTGCCATCCAGGCTGTGCT-3′ 5′- GGCACCTGAAACGCTCAGCTCCT-3′	375

PCR. PCR conditions were as follows: 25 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s for the actin cDNA clone; and 30 cycles of 94°C for 30 s, 55~62°C for 30 s depending on clones, and 72°C for 30 s for other cDNA clones. A partial cDNA fragment of the actin gene was obtained from both species by RT-PCR using degenerate primers, 5′-GARAARATGACNCARATHATG-3′ and 5′-TCNACRTCRCAYTTCATDAT-3′. After the PCR products were sequenced, primers specific for the actin gene were designed (Table 1). The constitutively expressed actin mRNA was used as a control. PCR products were separated on a 1.2% agarose gel, and the gel was photographed using a digital imaging system.

DNA Sequencing and Sequence Analysis

The nucleotide sequences of PCR products cloned into pBluescript-T vector were determined using a directional vector-specific T7 primer. Sequence data were processed with Vector NTI 7 software (InforMax, Inc.) to remove the primer sequence, the polyA tail, and ambiguous sequences. By assembling the sequence data, overlapping cDNA clones were excluded from further analysis. The BLASTn and BLASTx algorithms in the NCBI database (http://www.ncbi.nlm.nih.gov/database, accessed on Jan 13, 2009) were used to detect similarities between all edited sequences and previously deposited sequences. Similarities to known sequences were considered significant when the *E* values were less than 10^{-4} for sequences of more than 50 nucleotides in length. Finally, the unique sequences reported in this paper were deposited in NCBI's GenBank database under the accession numbers provided in Tables 2 and 3.

Results and Discussion

Analysis of Differentially Expressed Genes in Flowers of Two *Calanthe* Species

Modulation of floral development and of metabolic pathways related to shape, color, fragrance, and lifespan are

Table 2 List of clones upregulated in *C. discolor* flower buds and the results of a BLAST homology search

Clone	Accession	Size (bp)	Organism and cDNA homology (Accession number)	Score	*E* value	Identity
Cd001	GT066311	436	*C. melo* downward leaf curling protein (AB375061.1)	147	5e-32	77%
Cd002	GT066312	233	*S. tuberosum* clone PCM7 calmodulin gene (U20296.1)	75.2	1e-10	97%
Cd003	GT066313	340	*P. trichocarpa* clone POP027-K15 (AC215628.1)	78.8	2e-11	73%
Cd004	GT066314	186	No significant similarity			
Cd005	GT066315	737	No significant similarity			
Cd006	GT066316	558	*L. albus* mRNA for ATP citrate lyase (AJ344107.1)	343	4e-91	80%
Cd007	GT066317	748	*A. deliciosa* XTH4 (EU494949.1)	147	9e-32	72%
Cd008	GT066318	552	*Z. mays* calmodulin (NM_001159179.1)	176	1e-40	77%
Cd009	GT066319	394	*T. aestivum* thioredoxin H (AF286593.2)	134	3e-28	74%
Cd010	GT066320	814	*T. aestivum* RNA-binding protein (AF315811.1)	259	2e-65	72%
Cd011	GT066321	672	*H. vulgare* germin-like protein 6a (DQ647625.1)	340	7e-90	74%
Cd012	GT066322	251	*N. tabacum* type 2 proly 4-hydroxylase (AB471926.1)	114	2e-22	81%
Cd013	GT066323	200	No significant similarity			
Cd014	GT066324	828	*Z. mays* clone 225850 pnFL-2 mRNA (EZ057794.1)	102	2e-18	84%
Cd015	GT066336	653	*P. coarctata* histone H3 (AF109910.1)	495	1e-136	86%
Cd016	GT066325	636	*G. conopsea* AtPH1-like protein (EF051322.1)	320	6e-84	77%
Cd017	GT066326	336	No significant similarity			
Cd018	GT066327	472	No significant similarity			
Cd019	GT066328	205	No significant similarity			
Cd020	GT066329	531	No significant similarity			
Cd021	GT066330	248	No significant similarity			
Cd022	GT066331	293	*Z. mays* zinc finger protein LSD2 (EU976486.1)	68	3e-08	76%
Cd023	GT066332	373	*V. vinifera* hypothetical protein (XM_002285497.1)	154	3e-34	74%
Cd024	GT066333	624	*D. crumenatum* S-adenosylmethionine synthase (AF420238.1)	539	1e-130	87%
Cd025	GT066334	547	*Z. mays* flavonoid 3′-hydroxylase (NM_001157846.1)	224	1e-28	71%
Cd026	GT066335	398	*S. bicolor* hypothetical protein (XM_002448163.1)	154	3e-34	86%

tightly regulated by endogenous and environmental factors, which undoubtedly involve complex combinatorial gene expressions and specific function of genes (reviewed in Tanaka and Ohmiya 2008; Mondragón-Palomino and Theissen 2009). Although floral development and the acquisition of floral traits have been extensively characterized in model species, such as rice and *Arabidopsis*, they have yet to be deciphered in orchids, which produce complex flowers that differ markedly from those of all other plant families.

To investigate the regulatory mechanisms underlying orchid flower development and the metabolic pathways that dictate floral traits, we compared the expression pattern of genes in the floral organs of two closely related *Calanthe* species, *C. discolor* and *C. sieboldii*. While two species show high similarities in vegetative morphology, the flowers differ in terms of color, size, shape, and fragrance. Since gene expression can be influenced by subtle changes or differences among individuals and/or by environmental conditions, RNA was extracted from pools of flower buds derived from five individuals of each species. For an accurate and reproducible analysis, we employed annealing controlled primer (ACP)-based RT-PCR technology (Kim et al. 2004) to reduce false-positive signals resulting from the nonspecific annealing of short arbitrary primers. Using 120 ACPs and anchored oligo-dT (dT-ACP2) primer combinations, a total of 110 differential PCR fragments were observed on the agarose gels. Representative gel images are shown in Fig. 1. Of these, PCR bands consisting of more than 150 nucleotides of cDNA were excised from gels and cloned into the pBluescript-T vector. Clones harboring less than 150 nucleotides were excluded from further analysis since we found that the short cDNA fragment from 3′-end of polyA tail generally showed no significant similarity in a BLAST search analysis. In some cases, a second round of PCR amplification was performed to yield a sufficient amount of DNA for the cloning. The nucleotide sequences of the cDNA fragments cloned into the pBluescript-T plasmid were determined by single-pass sequencing using the vector-specific T7 primer. After removing poor-quality sequence data and overlapped sequences, 26 DEGs from *C. discolor* and 40 from *C. sieboldii* were obtained (Tables 2 and 3, respectively). A BLAST homology search revealed that

Table 3 List of clones upregulated in *C. sieboldii* flower buds and the results of a BLAST homology search

Clone	Accession	Size (bp)	Organism and cDNA homology (accession number)	Score	*E* value	Identity
Cs001	GT066337	557	*V. vinifera* thaumatin-like protein (DQ406688.1)	156	1e-34	77%
Cs002	GT066338	480	No significant similarity			
Cs003	GT066339	400	*S. bicolor* hypothetical protein (XM_002448163.1)	154	3e-34	70%
Cs004	GT066340	415	*P. trichocarpa* 4-coumarate-CoA ligase (NM_001065908.1)	138	4e-03	75%
Cs005	GT066341	691	*Z. mays* polygalacturonase-1 (EU956644.1)	266	7e-68	72%
Cs006	GT066342	636	*E. guineensis* annexin P35 (EU284950.1)	212	2e-51	69%
Cs007	GT066343	247	*G. tenera* granule-bound starch synthase (EF584708.1)	53.6	5e-04	96%
Cs008	GT066344	235	*S. lycopersicum* cDNA (AK323702.1)	55.4	1e-04	81%
Cs009	GT066345	754	*V. vinifera* hypothetical protein (XM_002271389.1)	62.6	3e-06	74%
Cs010	GT066346	319	*V. vinifera* hypothetical protein (XM_002269457.1)	120	6e-26	81%
Cs011	GT066347	762	*Z. mays* palmitoyl-protein thioesterase (EU963520.1)	315	3e-82	74%
Cs012	GT066348	699	*M. sativa* S-adenosylmethionine synthase (AY560003.1)	428	2e-116	82%
Cs013	GT066349	217	hypothetical (BT072556.1)	59	1e-05	92%
Cs014	GT066350	346	*A. longissima* 16.9 kDa heat-shock protein (AM709746.1)	59	1e-05	80%
Cs015	GT066351	627	*E. guineensis* IAA1 (AY556421.1)	333	1e-87	78%
Cs016	GT066352	702	No significant similarity			
Cs017	GT066353	220	No significant similarity			
Cs018	GT066354	1053	*O. sativa* cDNA clone (CT832628.1)	315	4e-82	70%
Cs019	GT066355	588	*V. vinifera* GTPase activating protein (XM_002270254.1)	156	1e-34	69%
Cs020	GT066356	455	*A. arguta* myo-inositol-1-phosphate synthase (AY005128.1)	242	9e-61	85%
Cs021	GT066357	440	*O. Gower* Fibrillin-like protein (AY940148.1)	482	6e-133	86%
Cs022	GT066358	390	*V. vinifera* hypothetical protein (XM_002280802.1)	134	3e-28	80%
Cs023	GT066359	357	*E. helleborine* mannose binding lectin (EHU07787)	228	2e-56	83%
Cs024	GT066360	564	*S. bicolor* hypothetical protein (XM_002463011.1)	71.6	4e-09	73%
Cs025	GT066361	636	*S. italica* DnaJ-like protein (DQ916829.1)	370	4e-99	78%
Cs026	GT066362	181	No significant similarity			
Cs027	GT066363	164	No significant similarity			
Cs028	GT066364	422	*Z. mays* NADH-ubiquinone oxidoreductase (NM_001157900.1)	219	1e-53	80%
Cs029	GT066365	526	*G. conopsea* glutathione transferase-like (EF051342.1)	185	2e-43	76%
Cs030	GT066366	143	No significant similarity			
Cs031	GT066367	618	*S. bicolor* hypothetical protein (XM_002446012.1)	293	8e-76	74%
Cs032	GT066368	567	No significant similarity			
Cs033	GT066369	584	*P. sativum* xyloglucan fucosyltransferase (AF223643.2)	264	4e-67	77%
Cs034	GT066370	820	*T. aestivum* GTPase SAR1 (EU660485.1)	596	6e-167	82%
Cs035	GT066371	268	*H. orientalis* heat shock protein 82 (AY389747.1)	311	1e-81	84%
Cs036	GT066372	844	*A. officinalis* xyloglucan endotransglycosylase (AF223420.1)	513	6e-142	75%
Cs037	GT066373	188	No significant similarity			
Cs038	GT066374	173	No significant similarity			
Cs039	GT066375	437	*P. glaucum* low temperature responsive protein (AY823550.1)	143	6e-31	81%
Cs040	GT066376	434	No significant similarity			

48 clones (72%) exhibited significant similarity to known genes from other organisms. However, 18 clones (28%) had no significant similarity to any sequences in the public database. This might be due to the absence of sequence information or due to the unique genes within *Calanthe* species. Taken together, our analysis suggests that a wide range of genes, potentially involved in diverse functions, is differentially expressed in floral organs of these two *Calanthe* species.

Confirmation of *DEG*s by Gene-specific RT-PCR

Semiquantitative RT-PCR using gene-specific primers was performed to confirm the differential expression patterns of

14 representative DEG clones. Since most clones are partial fragments corresponding to the 3′ region of cDNA, which contains the polyA tail and a high AT content, cycle parameters and annealing temperatures of the RT-PCR were optimized independently for each clone. All primers could be used to amplify the target mRNA from both species. RT-PCR results demonstrated that the differential expression patterns were reproducible for the tested clones.

A significant proportion of DEGs from this study share similarity with genes that encode enzymes involved in primary and secondary metabolism, hormonal pathways, and signal transduction pathways. The phenylpropanoid pathway contributes to the synthesis of thousands of compounds, including flavonoids, such as flavonols, anthocyanidins, and tannins (Hamberger and Hahlbrock 2004), and is known to be involved in the control of floral fragrances (Knudsen et al. 1993). The 4-coumarate:CoA-ligase protein (4CL, EC 6.2.1.12) catalyzes the conversion of hydroxycinnamic acids to hydroxycinnamoyl-CoA thioesters, precursors of a variety of phenylpropanoid biosynthetic derivatives. The *Cs004* clone shares high-sequence similarity with a gene that encodes 4-coumarate:CoA-ligase, and this gene is preferentially expressed in the floral buds of *C. sieboldii* and only slightly expressed in those of *C. discolor* (Figs. 1 and 2). Since *C. sieboldii* produces uniformly yellow color flower with a stronger fragrance than those of *C. discolor*, the implication of *Cs004* regarding to these phenotypes remains to be interested. Furthermore, we identified the *Cd025* clone, which encodes

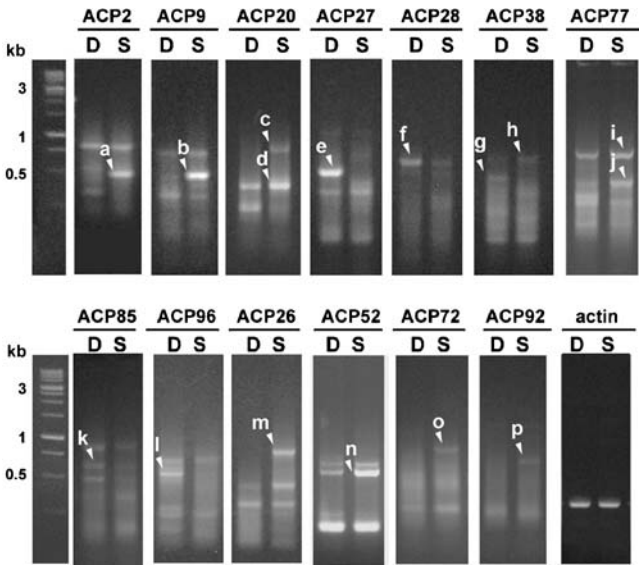

Fig. 1 Representative agarose gel images of DD RT-PCR analysis. Total RNA from flower buds of *C. discolor* (*D*) and *C. sieboldii* (*S*) were used for RT-PCR using ACPs (SeeGene, Inc.), as described in "Materials and Methods." The actin gene was amplified as a control. Cloned DEGs are labeled as follows: *a* Cs003; *b* Cs004; *c* Cs006; *d* Cs007; *e* Cd006; *f* Cd007; *g* Cd008; *h* Cs015; *i* Cs019; *j* Cs020; *k* Cd024; *l* Cd025; *m* Cs012; *n* Cs021; *o* Cs034; and *p* Cs039

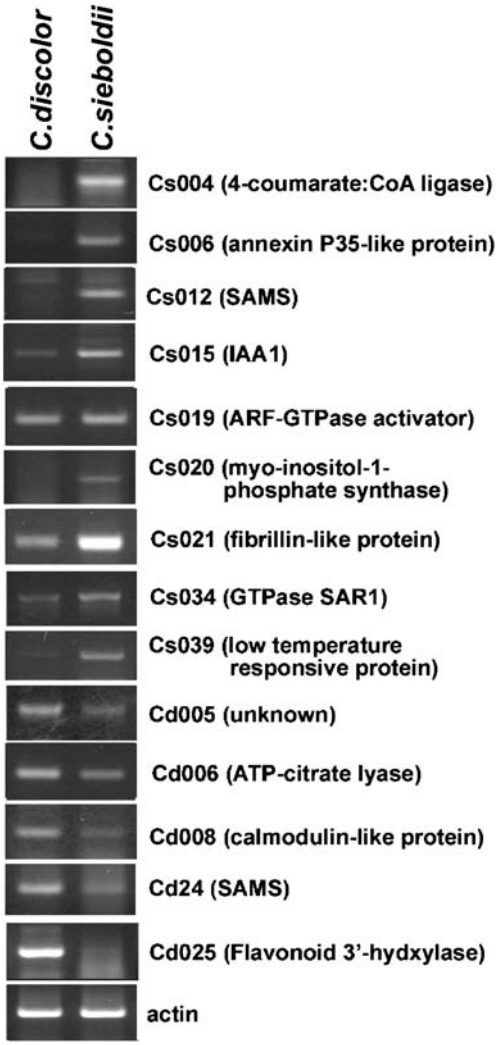

Fig. 2 Semiquantitative RT-PCR analysis of representative clones obtained by DD RT-PCR analysis. RT-PCR was performed using the gene-specific primers listed in Table 1. Clone names are represented by the protein names that showed the highest similarity in a BLAST homology search. Actin was used as the internal control for RT-PCR

a protein with high sequence similarity to flavonoid 3′-hydroxylase (F3′H), a member of the cytochrome P450 family that controls flower color. Unlike the *Cs004* clone, its expression was higher in floral buds of *C. discolor* than in those of *C. sieboldii* (Figs. 1 and 2), suggesting its possible roles in the biosynthetic pathways contributing to floral phenotypes in *C. discolor*.

S-adenosylmethionine synthase (SAMS, EC 2.5.1.6) is a key enzyme in the biosynthesis of polyamines and ethylene (Yang and Hoffman 1984) that catalyzes the biosynthesis of SAM from Met and ATP. It has been reported that *SAMS* genes play critical roles during ovary development and floral senescence and also in the plant's response to treatment with phytohormones (Gómez-Gómez and Carrasco 1998). Interestingly, the *Cd024* and *Cs012* clones, which encode putative SAMS, were found to be differen-

tially expressed, with greater expression in the floral buds of *C. sieboldii* and *C. discolor*, respectively (Figs. 1 and 2). Further sequence analysis using the PCR products demonstrated that a high level of species conservation of *Cs012* and *Cd024* exists between two species, as both the nucleotide and deduced amino acid sequences share 99.5% and 100% identity, respectively (Fig. 3a). However, the nucleotide and deduced amino acid sequences of *Cd012* and *Cd024* clones share about 73% and 85% identity with each other, respectively (Fig. 3b), suggesting that these two clones belong to isoforms of putative *SAMS* gene family. Therefore, it is interesting to evaluate that the unique expression patterns of these genes may impact on metabolic regulation during floral development in each of the *Calanthe* species.

The clones *Cs015*, *Cs019*, *Cs034*, *Cd002*, and *Cd008* showed strong similarity to genes encoding indole-3-acetic acid 1 (IAA1), ARF, SAR GTPases, and two calmodulin isoforms, respectively. The functions of these proteins in controlling floral development are largely unknown at present. However, auxin plays an essential role in the initiation of floral primordia and organ outgrowth, the identity of floral organs, and the development of the female reproductive organs of angiosperms (Vernoux et al. 2000;

A

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dCs012  (1)   TCGGGAAAGGACCCGACTAAGGTCAATCGTAGCGGCGCCTACATTGTGAG
sCs012  (1)   .......................G.........................
dCd024  (1)   ..C..C.....T.....A......G.CA.G.....T..G..T..C..A..
sCd024  (1)   ..C..C.....T.....A......G.CA.G.....T..G..T..C..A..

dCs012  (51)  GCAGGCTGCTAAGAGCATCGTCGCTAATGGATTTGCGAGGAGATGCATCG
sCs012  (51)  .................................................
dCd024  (51)  ...A..G..G......G.T..G..C..C...C.C..TC.CC.C.....T.
SCd024  (51)  ...A..G..G......G.T..G..C..C...C.T..TC.CC.T.....T.

dCs012  (101) TCCAAGTGTCGTATGCCATTGGCGTGCCTGAGCCATTGTCGGTGTTTGTC
sCs012  (101) .................................................
dCd024  (101) .T..G..T..............T..T..C......C.C..T.....C..T
sCd024  (101) .T..G..T..............T..T..C......C.C..T.....C..T

dCs012  (151) GATACATACGGTACTGGAAAGATTTCTGATAAGGAGATACTTGAGATTGT
sCs012  (151) .................................................
dCd024  (151) ..C..C........G..C.....CC.A...........C..GA.T.....
sCd024  (151) ..C..C........G..C.....CC.A...........C..GA.T.....

dCs012  (201) TAAGAAGAGTTTTGATTTCAGGCCTGGAGTGATTAGCATCAACCTTGATC
sCs012  (201) ........................G........................
dCd024  (201) G...G...A......C........G..GA......C.....C..C.
sCd024  (201) G...G...A......C........G..GA......C...T.....C..C.

sCs012  (251) TGAAGCGCGGTGGTAATGGGAGATTCTTGAAGACTGCTGCTTATGGTCAT
sCs012  (251) .................................................
dCd024  (251) .....AAG..C..C..CC..    ...A.C.....G........C..G..C
sCd024  (251) .....AAG..C..C..CC..    ...A.C.....G........C..G..C

dCs012  (301) TTTGGAAGGGATGATGCTGACTTCACCTGGGAGGTAATTAAGCCTCTCAA
sCs012  (301) .................................................
dCd024  (298) ........A..C..CC....T..............GG.G...T.C.....
sCd024  (298) ........A..C..CC....T..............GG.G...T.C.....

dCs012  (351) GTATGATGGAAAGTCTGCAGCT
sCs012  (351) ......................
dCd024  (348) ..GG..GAA.CC.G.G...
sCd024  (348) ..GG..GAA.CC.G.G...
```

B

```
Cs012 (1)   SGKDPTKVNRSGAYIVRQAAKSIVANGFARRCIVQVSYAIGVPEPLSVFV
Cd024 (1)   ........D.............V....L......................


Cs012 (51)  DTYGTGKISDKEILEIVKKSFDFRPGVISINLDLKRGGNGRFLKTAAYGH
Cs012 (51)  ..............N...EN......M.T......K... ..I.......

Cs012 (101) FGRDDADFTWEVIKPLKYDGKSAA
Cd024 (100) .....P......V.S..WEKPA.
```

Fig. 3 Alignment of the nucleotide (**a**) and deduced amino acid (**b**) sequences corresponding to the coding regions of the Cs012 and Cd024 clones. Differences in sequence between the two species are represented in *shadow boxes*. *dCs012* and *sCs012* indicate the *Cs012* clone isolated from each *C. discolor* and *C. sieboldii*, respectively. *dCd024* and *sCd024* indicate the *Cd012* clone isolated from each *C. discolor* and *C. sieboldii*, respectively. *Asterisks* indicate the *S*-adenosylmethionine synthetase C-terminal domain (pfam02773) with *E* value of 8e-44 identified by NCBI conserved domain search

Nemhauser et al. 2000; Okada et al. 1991; Pfluger and Zambryski 2004). The IAA1 is believed to have a role in auxin signal transduction. GTPase and calmodulin are known to act as upstream regulators in diverse signal transduction pathways (Memon 2004; Yang and Poovaiah 2003). The differential expression pattern of these genes (Figs. 1 and 2) is of great interest, since it can help in assigning their possible functions in floral development of two *Calanthe* species.

Although the subset of genes identified in this study makes an initial contribution to the field of functional genomics in *Calanthe* orchids, the identification of differentially expressed genes may help in the understanding of metabolic pathways possibly involved in flowering and flower architecture, biosynthesis of pigments and fragrances, and the lifespan of flowers. The described genes in this study are currently being studied in search for candidates that might regulate floral development allowing us to step forward in the genetic manipulation of *Calanthe* species. Therefore, further research into the biological functions of these genes may greatly enhance our understanding of floral development and contribute to the pool of available plant biotechnology resources.

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