

Full Length Research Paper

Differential expressions of putative genes in various floral organs of the Pigeon orchid (*Dendrobium crumenatum*) using GeneFishing

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Accepted 18 May, 2009

Nine Differentially Expressed Genes (DEGs) were detected in the five floral organs of *Dendrobium crumenatum* and three DEGs of relatively high expression in the column, were significantly homologous to the small Heat Shock Protein (HSP) differentially regulated during pollen development and heat stress in tobacco (DEG3-8), pectin methylesterase enzyme (PME) which was a male-flower specific gene in *Salix gilgiana* (DEG6-1) and the 14-3-3 protein which was differentially expressed and upregulated in *Malus x domestica* (DEG9-9) during fruit ripening. Generally, the differential expressions of the DEGs in the column and other floral organs were distinct, indicating the suppressive role of column DEGs on other floral organ DEGs. Interactions between the other floral DEGs (excluding column DEGs) indicated pattern expression specificity to each floral organ. Following this investigation, further molecular expression investigations are required and essential to develop and establish an orchid floral ontogenic model.

Key words: *Dendrobium crumenatum*, Differentially Expressed Genes (DEGs), floral organs.

INTRODUCTION

The floral developments of orchids are unique and more complicated than the understanding of conventional monocotyledonous flowers. Orchid flowers are borne on stalks called pedicels, which rotates a 180° during the growth and development of the flower (a phenomenon known as resupination), resulting in the mature orchid flower being borne upside down (Ernst and Arditti, 1994).

As opposed to the general Angiosperm floral model, an orchid floral model was specifically illustrated by Darwin, as consisting of three sepals, three petals and six anthers in two whorls and three pistils. Among the petals, there is one unique petal (larger-sized, differently coloured and elaborately-shaped) known as the labellum that it is ontogenically believed to be formed from one petal and two petaloid stamens of the outer whorl, where the crests or

ridges were derived from the stamens (John, 2003).

The sexual organs (pistil and stamen) are fused together into a structure called the column, believed to be derived from three pistils and generally of four stamens (one from the outer and three from the inner whorl). Among the pistils, one forms the rostellum, while the remaining bottom two are confluent. Generally, orchid floral parts do not completely follow the general ABC model (Bowman et al., 1989; Carpenter and Coen, 1990; Schwarz-Sommer et al., 1990; Bowman et al., 1991; Weigel and Meyerowitz, 1994).

Despite the physical observation of orchid floral morphology and deduction of various ontogenic theories, the study of molecular interactions and conceived genetic models specifying floral organ identity, would most importantly develop and establish the orchid floral ontogeny and even the development of seed production. However, the identification and molecular expressions of flowering-time and meristem identity genes of the orchid family, leaves much to be elucidated.

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The DEG technique has been successful in identifying and assessing gene functions of various metabolic events in various orchid species: early flowering, carbohydrate metabolism and stress response physiology in *Oncidium* (Tan et al., 2005) floral transition, transcriptional regulation, cell division and respiration in *Dendrobium Madame Thong-In* (Yu and Goh, 2000); pollinated ovary in *Cymbidium hybridum* (Chen et al., 2006); benzylaminopurine (BA) induction in *D. Sonia* flower buds (Yang et al., 2003); and flower buds of wild and peloric in *Phalaenopsis* (Chen et al., 2005).

The Pigeon orchid (*D. crumenatum*) is a tropical epiphytic orchid that demonstrates a flowering process associated with temperature changes. The developed meristem enters a phase of dormancy and continued floral development is believed to be triggered by a sudden drop of temperature of about 10°C. The differentiation of meristem and tissue follows. Almost a week after stimulation, the formed floral buds would bloom simultaneously. The white flowers consists of sepals (elliptical dorsal and two lateral sepals), petals (two lateral petals similar to dorsal sepal and one labellum of three lobes), and the column (pistils and stamens) (Seidenfaden and Wood, 1992).

However, conventional genetic models could not establish the molecular regulation of flower development in this species due to the limited availability of relevant genes and expressions in the orchid species. Therefore in this communication, the expression profile of putative floral organ specific transcripts from the Pigeon orchid is characterized using the DEG technique.

MATERIALS AND METHODS

Sample collection and total RNA isolation

Samples of fully opened flowers, developing flower buds 3-4 days before anthesis, roots (RT) and stems (ST), were collected from wild Pigeon orchids (*D. crumenatum*) that were growing at the University Putra Malaysia campus in Serdang, Selangor, Malaysia. The mature flowers were dissected and separated into five different parts: dorsal sepal (DS), lateral sepal (LS), petal (PT), lip (LP) and column (WC; including anther cap, pollinia, stigma, ovary, column and column foot). All samples were briefly frozen in liquid nitrogen and kept at 80°C until use. Total RNA was extracted from floral organs according to Champagne and Kuehnle (2000) with some modification (Prescott and Martin, 1987; Schultz et al., 1994).

Generally, PVP was used to eliminate polysaccharides, flavonoids and other contaminating compounds and chloroform extractions were performed prior to using phenol. The incubation period for RNA precipitation with lithium chloride (LiCl) was reduced from overnight to two hours. For non-floral organs, total RNA was isolated according to Rochester et al. (1986).

GeneFishing and cloning

For first-strand cDNA synthesis, 3 µg of total RNA from each organ were mixed with dT-ACP1 [5'CTGTGAATGCTGCGACTACGATIIIIIT (18)-3'], to a final concentration of 1 µM and adjusted to a volume of 10.5 µl with DEPC-treated water in 5 separate reaction

tubes. Each tube were added to a final volume of 20 µl with the final concentration of 1x RT buffer, 0.5 mM dNTP, 20 U RNase inhibitor (Promega) and 200U MMLV Reverse Transcriptase (Seegene). The reaction was performed for 90min at 42°C before heating at 94°C for 2 min to inactivate the reaction.

First-strand cDNAs were chilled on ice for 2 min before being centrifuged briefly and stored at 20°C. The second-strand cDNA synthesis was performed in a reaction containing 5 µl of the 5x diluted first-strand cDNAs, 0.5 µM Arbitrary ACP (one of the 10 arbitrary ACPs provided the GeneFishing™ kit), 0.5 µM dT-ACP2, 1x SeeAmp™ ACP™ Master Mix and topped up to a final volume of 20 µl with distilled water. Incubation of the mixture was initially performed at 94°C for 5 min, 50°C for 3 min and 72°C for 1 min for a single cycle, followed by 40 cycles of 94°C for 40 s, 65°C for 40 s and 72°C for 40 s, after which a post extension was performed at 72°C for 5 min.

The PCR products were then subjected to electrophoresis on 1.2 to 2.0% (depending on PCR product size) agarose gel and stained with 1 µg/ml ethidium bromide. Potential DEGs were identified and excised from the agarose gels with a clean scalpel and purified using QIAquick Gel Extraction Kit (Qiagen).

The amplified potential DEG inserts were subsequently cloned into the pCR®2.1-TOPO® vector (Invitrogen, USA) according to manufacturer's instructions. Ten positive clones for each DEG insert were isolated and the insert sizes were verified by PCR and sequenced using universal primers.

Clone identity was determined according to sequence homology with the National Centre for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/>). The BLASTX algorithm was used for similarity search against the translated amino acid sequences in the database. The homology was deemed significant when the E-value is lesser than or equal to 1e⁻⁵ (Gish and States, 1993). Sequences were aligned with several closely related proteins using CLUSTALW, BioEdit version 5.0.6.

Semi-quantitative RT-PCR and Southern hybridization

Subsequently selected DEGs expressions (DEG3-8, DEG6-1, DEG9-9) were further analysed in the five floral organs by semi-quantitative RT-PCR and Southern Hybridization. The Cyclophilin (Cyp) housekeeping gene homologue from the Pigeon orchid was used as internal control and normalization of starting materials (Nicot et al., 2005; Brunner et al., 2004). The Cyp gene was isolated from total RNA (of the column) using degenerate primers (Choong, 2004) using Quantitect Reverse Transcription Kit (Qiagen, USA) cloned into the pCR®2.1-TOPO® vector (Invitrogen, USA) according to manufacturer's instructions; and sequenced.

Total RNA samples used for first-strand cDNA synthesis (2 µg) and first-strand cDNA samples used (≈10 ng/µl) for PCR amplification were normalized. Forward and reverse Gene Specific Primers (GSP) were designed for analysis of selected DEG clones using the Primer Premier 5.0 software (Table 1).

First-strand cDNA samples from the five floral organs were used for the semi-quantitative RT-PCR analysis. The 20 µl semi-quantitative RT-PCR reaction mix that was assembled on ice contained: 2 µl 10X Bioron Complete buffer, 0.25 µl dNTP mix (10 mM each); 0.40 µl each of forward and reverse primers (5 µM); 0.05 µl of SuperHot Taq DNA polymerase (5 U/µl) (Bioron); 5X diluted cDNA sample (≈10 ng/µl); and sterile distilled water to complete a total volume of 20 µl. Denaturation of template was carried out at 95°C for 1 min, followed by 33 cycles of amplification (denaturation at 95°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s) and a final extension of 72°C for 30 s.

PCR products were electrophoresed on 1.5% TAE agarose gels, stained with ethidium bromide (EtBr) for gel visualization, blotted onto nylon membrane and hybridized with biotinylated clone speci-

Table 1. GSP sequences and RT-PCR conditions for analysis of DEG clones.

Clone	Direction	GSP sequences	RT-PCR conditions
DEG3-8	Forward Reverse	5' TGGTAAACGGTGTGCTCA 3' 5'ATAACACACAGGTTGCAA 3'	95°C - 15 s 55°C - 30 s 72°C - 30 s (33 cycles)
DEG6-1	Forward Reverse	5' GCTTTTGATTCGTTTGGG 3' 5'GTGAAAACCTGTTGGAAATGC 3'	
DEG9-9	Forward Reverse	5' CTCTGTTTTAATGACTGGACTG 3' 5' ATTGAGCAGTAATAGTGGAGAC 3'	
Cyp (Choong, 2004)	Forward Reverse	5' GCTGG(A/C/G/T)CG(A/C/G/T)(A/G)TGGTGATGGAGCT-3' 5' GCGGT(C/T)CAGATGAA(A/G)AACTGAGA-3'	

fic probes (biotin-21-dUTP) according to Sambrook and Russell (2001).

Quantitative Real-time RT-PCR

For quantitative real-time RT-PCR, the same GSPs for clones DEG6-1 and DEG9-9 were used (Table 1). However, GSPs for clone DEG3-8 (dDEG3-8) and the Cyp homologue (dCyp) was redesigned to improve PCR efficiency and specificity (Table 2).

First-strand cDNA samples from eight organs that were used for real-time analysis are the five floral organs, roots, stems and developing buds 3-4 days before anthesis. The reaction mixture for real-time RT-PCR contained 1X Incomplete PCR buffer (Bioron), 5 mM MgCl₂, 125 μM each of dNTPs, 200 nM each of forward and reverse gene specific primers, 0.5U SuperHot *Taq* DNA polymerase (5 U/μl) (Bioron), approximately 150 ng of first-strand cDNA, 0.50 μl of 1000X dilution SYBR (R) Green I dye (BioWhittaker Molecular Applications) and distilled water to a total volume of 20 μl per reaction. The cycling conditions were: 1 cycle for denaturation and "hot-start" purposes at 95°C for 3 min; followed by 30 four-segment cycles of amplification at 95°C for 20 s, primer specific annealing temperature for 30 s (55°C for GSPs DEG6-1, DEG9-9 and Cyp; 58°C for GSPs dDEG3-8 and dCyp) and extension at 72°C for 30 s; acquisition temperature at 82°C for 1 s; and a subsequent product melting curve analysis from 72°C to 98°C with a temperature transition rate of 0.2°C/s. All PCRs were conducted in triplicate and normalized to Cyp. Each of the normalized relative values were further divided by the value of the calibrator (column) and the relative expression level is presented as an n-fold expression differences compared to the calibrator organ, column. DS, dorsal sepal; LS, lateral sepal; PT, petal; LP, lip; WC, column; RT, root; ST, stem; DB, developing bud.

RESULTS

Detection of DEGs by amplification of cDNAs

We isolated total RNA from five different organs of the *D. crumenatum* and synthesized first-strand cDNAs that were amplified using four arbitrary ACP primers (ACP1, ACP2, ACP3 and ACP8) from the GeneFishing™ kit in combination with the dT-ACP2 primer. A total of nine potential DEGs were successfully amplified, electrophoretically separated and observed on agarose gels (Plate 1 and Table 1). Three DEGs were observed in RT-PCR products amplified with arbitrary primer ACP1: DEG-1; DEG-2; and DEG-3. DEG-1 was present in all sepals and

lip; DEG-2 was present in all sepals and petals including the lip; DEG-3 was present only in the column. Two DEGs were observed in RT-PCR products amplified with arbitrary primer ACP2: DEG-4 and DEG-5. DEG-4 and DEG-5 were present in all sepals and petals including lip except column. One DEG was observed in RT-PCR products amplified with arbitrary primer ACP3, DEG-6. DEG-6 was present only in the column. Three DEGs were observed in RT-PCR products amplified with arbitrary primer ACP8: DEG-7; DEG-8; and DEG-9. DEG-7 and DEG-8 were present in all sepals and petals excluding lip; and DEG-9 was only present in the column. Among these identified DEGs, DEG-1 (from the dorsal sepal), DEG-3 (column), DEG-4 (lip), DEG-5 (column), DEG-6 (column) and DEG-9 (column) were successfully excised, purified and cloned for subsequent sequencing.

Putative annotation of sequenced DEGs

A total of ten DEG clones were sequenced and subjected to BLASTX algorithm search against the protein database at NCBI. Most of the clones corresponded to known proteins, within the range of 42-78% homology, except for one unknown gene with a 88% sequence homology (Table 3). Among these putative DEG clones, three clones (DEG3-8, DEG6-1 and DEG9-9) showed high homology with genes specifically expressed in a particular organ or to those that are differentially regulated during specific developmental processes. These clones were selected for subsequent Southern hybridization and real-time RT-PCR detection and quantification in the five floral organs of *D. crumenatum*.

The DEG3-8 incomplete ORF nucleotide sequence of 601 bp, 154 amino acid polypeptide, has a significant homology to class-1 small HSPs from *N. tabacum* (75%) (AAR01523), *L. peruvianum* (75%) (CAA12387) and *A. comosus* (67%) (AAM28293) (Table 3 and Figure 1). This clone was truncated at the 5' region (Met codon was not found), complete at the 3' end, included a 3' untranslated region (UTR) and the GVLTV motif which belongs to part of the α-crystallin domain of the predicted protein. Highly conserved regions of the amino acid sequence, I and II, (Figure 1) was homologous to the common α-crystallin

Table 2. Redesigned GSP sequences for DEG3-8 (dDEG3-8) and Cyp (dCyp).

Clone	Direction	GSP sequences
dDEG3-8	Forward	5' CAGGCTGAGGAAAATGGC 3'
	Reverse	5' TAACACACAGGTTTCGCAA 3'
dCyp	Forward	5' GAGAAGGGCGTCGGCAAG 3'
	Reverse	5' TCCGTTGGTGTGGGTCC 3'

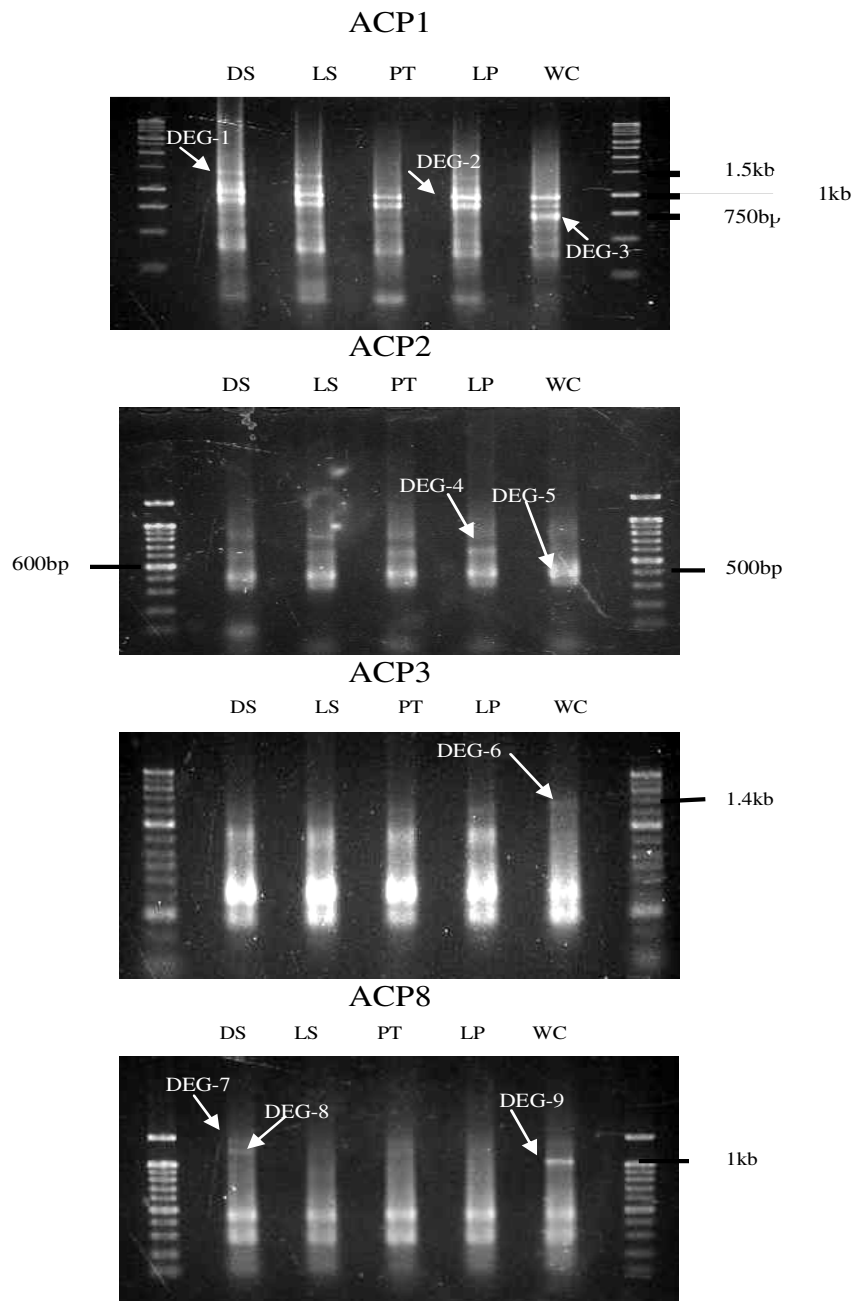
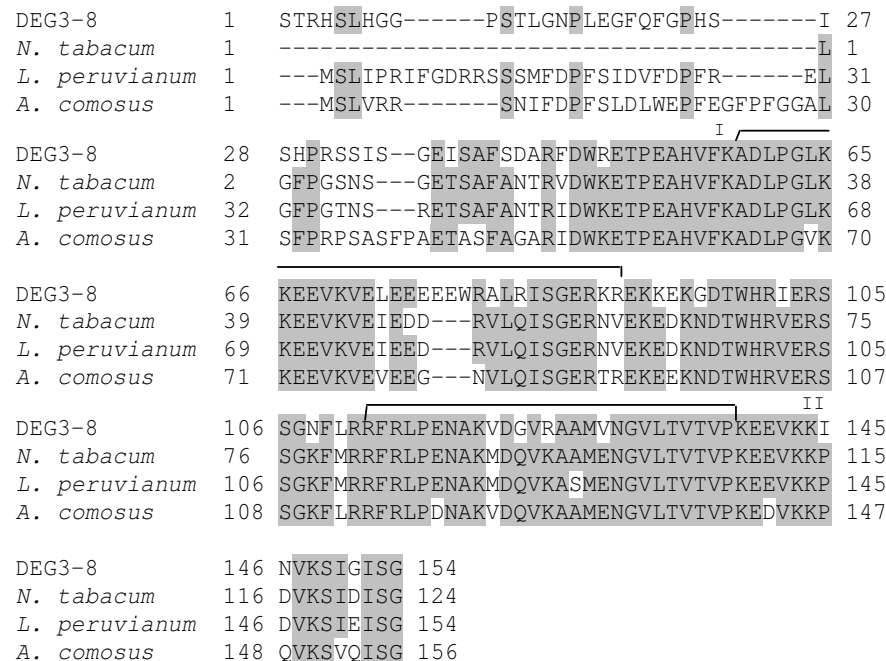
**Plate 1.** Gel electrophoresis of PCR products from dorsal sepal (DS), lateral sepal (LS), petal, (PT), lip (LP) and whole column (WC) using arbitrary ACP1, ACP2, ACP3 and ACP8 forward primers, and dT-ACP2 reverse primers, on 1.5% (w/v) agarose gel. Arrows indicate the position of selected DEGs; D E G-1 to DEG-9.

Table 3. Summary of DEG clones in floral organs [dorsal sepal (DS), lateral sepal (LS), petal (PT), lip (LP) and column (WC)] of the Pigeon orchid and their significant homology matches.

Putative clones	Similarity (Organism)	Putative Function / Expression	Accession	Identity	E-Value	Floral organ of <i>D. crumenatum</i>
DEG1-8	Papain-like Cysteine proteinase (<i>Gossypium hirsutum</i>)	Stress induced protein	CAE54306	74%	3e ⁻³⁷	DS, LS and LP
DEG3-3	Class-1 LMW heat shock protein (<i>Ananas comosus</i>)	Tissue-regulated, root development	AAM28293	42%	3e ⁻²²	WC
DEG3-7	Hsp20.1 protein (<i>Lycopersicon peruvianum</i>)	Stress induced proteins	CAA12387	51%	4e ⁻²⁴	WC
DEG3-8	Cytosolic class I small heat shock protein 3B (<i>Nicotiana tabacum</i>)	Differentially regulated during pollen development and heat stress	AAR01523	75%	4e ⁻³²	WC
DEG4-2	Acyl-activating enzyme 11 (<i>Arabidopsis thaliana</i>)	Lipid metabolism	AAP03024	59%	2e ⁻¹⁹	DS, LS, PT and LP
DEG4-9					1e ⁻¹⁹	DS, LS, PT and LP
DEG5-9	Unknown protein (<i>Arabidopsis thaliana</i>)	-	AAF13094	88%	7e ⁻²⁷	DS, LS, PT and LP
DEG6-1	Pectin methylesterase (<i>Salix gilgiana</i>)	Male-flower specific	BAA89480	62%	4e ⁻¹¹⁸	WC
DEG9-6	14-3-3 family protein (<i>Malus domestica</i>)	Differentially expressed and upregulated during fruit ripening	AAV50005	77%	4e ⁻⁶⁹	WC

**Figure 1.** Amino acid alignment of clone DEG3-8 with LMW HSPs from other plants. The predicted sequences of *Nicotiana tabacum* (GenBank accession no. AAR01523), *L. peruvianum* (GenBank accession no. CAA12387) and *Ananas comosus* (GenBank accession no. AAM28293) were aligned to display homology. Conserved amino acids in grey. I and II represents the highly conserved amino acid motifs (corresponding to the α -crystallin domain) in all LMW HSPs (Medina-Escobar et al., 1998). Gaps were automatically inserted to optimize alignment.

domain (De Jong et al., 1993). The observation further supports the identity of the cDNA insert within clone DEG3-8 as a putative partial clone of the low molecular weight heat shock protein (LMW HSP) from *D. crumenatum*.

The DEG6-1 incomplete ORF nucleotide sequence of 1281 bp, 342 amino acid polypeptide, has a significant homology to the conserved domain that signifies the pectin methylesterase (PME) gene family, a predicted pectinesterase domain from the PME gene of *D. crumenatum* (Table 3 and Figure 2). It was also homologous to the PME enzyme from *Salix gilgiana* (62%) (BAA89480), *Arabidopsis thaliana* (56%) (AAP40488) and *Zea mays* (52%) (CAA73733) (Figure 2).

The DEG9-9 incomplete ORF nucleotide sequence of 984 bp, 264 amino acid polypeptide (Table 3 and Figure 3), has a significant homology to conserved residues of the 14-3-3 family in *Malus x domestica* (AAV50005), *Lycopersicon esculentum* (P93211), *Solanum tuberosum* (P93784) and *Mesembryanthemum crystallinum* (P93259) (Figure 3). DEG9-9, the predicted gene product for 14-3-3 proteins was selected for further characterization of its potential regulation in *D. crumenatum* flowers.

Expression of the selected DEG clones in the five floral organs of *D. Crumenatum* via Semi-quantitative RT-PCR and real-time RT PCR

The selected DEGs were amplified in the five floral organs via semi-quantitative RT-PCR. Subsequently, the products were electrophoretically separated and detected via southern hybridization. As expected, all the selected DEGs were strongly expressed in the column, since the clones were exclusively expressed in and isolated from the column originally. However, there were relatively weak hybridization signals of DEG3-8 in the dorsal sepal and petal; DEG6-1 in all floral organs; and DEG9-9 in lateral sepal, petal and lip except the dorsal petals.

Real-time RT-PCR was also conducted to determine the efficiency of the GeneFishing™ technique in identifying DEGs since it is sensitive to low amounts of samples compared to the semi-quantitative PCR and Southern hybridization techniques. Furthermore, the analysis included the five floral organs (dorsal sepals, lateral sepals, petals, lip and columns) and non-floral organs (roots, stems) and floral buds (3-4 days before anthesis), to ascertain the expression specificity of the selected DEGs in floral organs. As expected, expressions of all the selected DEGs were observed in the floral column, consistent with initial GeneFishing™ screening and semi-quantitative RT-PCR analysis (Plate 1 and Table 3). However, none of the selected DEGs were detectable in both the non-floral organs, indicating that these DEGs were specific floral development genes (Figure 4). Furthermore, the expression of DEG9-9 was detected in developing floral buds. It was also noticed that real-time RT-PCR and the initial GeneFishing™ technique did not

detect DEG expressions in floral organs other than the column, as observed on the Southern blot of the semi-quantitative RT-PCR amplification technique (Plate 2) (DS, LS, PT and LP). This may be due to the low expression intensities of the DEGs compared to cyclophilin (Plate 2).

DISCUSSION

DEGs expressed in column vs. other floral organs

Nine DEG expressions (DEG1-DEG9) were detected in the five floral organs of *D. crumenatum* (Table 3). Generally, there was a distinct difference between DEGs expressed in columns and those expressed in the other floral organs, in that DEG3, DEG6 and DEG9 were observed to be exclusively expressed in the column, while DEG2, DEG4 and DEG5 expressions were observed in all floral organs except the column (Table 3). Although this may indicate that DEGs expressed in the column are column specific proteins, weak expressions of these DEGs were detected by Southern hybridization analysis of semi-quantitative RT-PCR in other floral organs.

This further implicates that column DEGs are associated with the suppression of DEGs of other floral organs and thereby down regulates their expression in the column. Therefore, it cannot be dismissed that DEG2, DEG4 and DEG5 may have been expressed in the column but was suppressed by column DEGs and thereby allow specification of the column organ to take place. Thus, the incorporation of these column-expressed DEGs and their interaction with other genes in floral ontogeny studies would contribute to the elucidation of their functional areas as meristem identity genes.

In contrast to DEGs expressed only in columns, both dorsal and lateral sepals showed DEG expressions from the remaining DEGs (DEG1, DEG2, DEG4, DEG5, DEG7 and DEG8), indicating that DEGs involved in sepal development are not involved in column development, due to possible suppression by column DEGs. Therefore, this implies that the presence or absence of column DEGs allowed the specific development of columns or sepals.

In the petal, all sepal DEGs were expressed except for DEG1, indicating that the absence or presence of DEG1 allowed the specific development of the petal or sepal respectively. In the lip, all sepal DEGs were expressed except for DEG7 and DEG8, indicating that the absence or presence of these DEGs allowed the specific development of the lip or petal respectively.

Putative functions of column DEGs

DEG3-8 was uniquely expressed in the column and BLAST analysis showed highest similarity to class-1 small HSPs. In tobacco, class-1 small HSPs from tobacco

DEG6-1	1	-----	1
<i>S. gilgiana</i>	1	MVFQDFDQLSERRRLERQOKLRKKII IASVSSIAFFVIVGAGVFSLVS--	48
<i>A. thaliana</i>	1	MAFQDFDKIQERNANRRKRFRKRI IVGTVSLLVVVAIVGGAFAVYAYE	50
<i>Z. mays</i>	1	-----MAKALLGGLSAILVVAVVVG-VVATVT--	26
DEG6-1	1	-----	1
<i>S. gilgiana</i>	48	-----NHDISSPENNNG-----SPSAATR-----PAEKAKPI	75
<i>A. thaliana</i>	51	KRNEQQQQQQAKNHNKSGSGNNVVKDSDKKSPSPPTPSQKAPVSAAQSV	100
<i>Z. mays</i>	26	-----RSGKKAGDNFT-----VPGEASLANVRQVGQVQVPR	56
DEG6-1	1	-----	1
<i>S. gilgiana</i>	76	S--HVARVIKTVCNATTYQDTCQNTLEKGVLGKDPSSVQPKDLLKIAIKA	123
<i>A. thaliana</i>	101	KPGQGDKI IQTLCSSSTLYMQICEKTLKN-RTDKGFALDNPTTFLKSAIEA	149
<i>Z. mays</i>	57	AHPVQGVVREDVVP GHQWHREPQGGVQP--RGQGGAGVGDGGRVAVQVDR	104
DEG6-1	1	-----	1
<i>S. gilgiana</i>	124	ADEEIDKVIKKASSFKFDKPREKAAFDDCLELIEDAKEELKNSVDCIG-N	172
<i>A. thaliana</i>	150	VNEDLDLVLEKVL SLKTENQDDKDAIEQCKLLVEDAKEETVASLNKINVT	199
<i>Z. mays</i>	105	RGQGSDSMTESAR-----EDCKLLEDAADDLRGMLEMAG-G	140
DEG6-1	1	-----XYQAF	6
<i>S. gilgiana</i>	173	DIGKLANAPDLSNWSAVMSYQOTCIDGFFPEGKLSDMKETFKATRELT	222
<i>A. thaliana</i>	200	EVNSFEKVVDPDLESWLSAVMSYQETCLDGFEEGNLSEVKTSVNSSQVLT	249
<i>Z. mays</i>	141	DIKVLFSRSDLETWLTGVMTFMDTCVDFGVDEKCLKADMHSVVRNATELS	190
DEG6-1	7	S-----WGP	10
<i>S. gilgiana</i>	223	SNSLAMVSSLVSLKNSF---SGLNRRLLAEEQNSPSLDKDGVPQWMS	269
<i>A. thaliana</i>	250	SNSLALIKTFTENLS--PV--MKVVERHLL-----DGIPSWVS	283
<i>Z. mays</i>	191	SNALAITNSLGGILKKMDLGMFSKDSRRRLLSSEQ-----DEKGWVWMR	235
DEG6-1	11	EDERRVLNQVNTN-LKPNVTAKDGTGDYTAISMALAKMPEEYSGRYVIY	59
<i>S. gilgiana</i>	270	HEDRRILKGADKDKPKPNVSVAKDGSDFKTI SEALAAMPKYEGRYVIF	319
<i>A. thaliana</i>	284	NDDRRMLRAVDVKALKNATVAKDGSDFTTINDALRAMPEKYEGRYIY	333
<i>Z. mays</i>	236	SPERKLLASGNQP--KPNIAIVAKDGSQFQSIQQAVDAVFKGHQGRYVIY	283
DEG6-1	60	VKEGVYEETVNVTKQPNLTIYDGGAKTITGEKNFVDGVRTFMATFV	109
<i>S. gilgiana</i>	320	VKQGVYDETVTVTKKMANITMYGDGSQKTI VGTGNKNFADGVQTFRTATFA	369
<i>A. thaliana</i>	334	VKQGIYDEYVTVDKKANLTMVGDGSQKTI VGTGNKSHAKKIRTFLTATFV	383
<i>Z. mays</i>	284	VKAGLYDEIVMVPKDKVNIIFYMGDGPQSRVTRKSFADGITTMKTATFS	333
DEG6-1	110	VSGDGMGIGLGVNRNTAGAIKHQAVAIRVQSDRSIFFECRFEGYQDTLYA	159
<i>S. gilgiana</i>	370	VLGDGFLCKFMGFRNTAGPEKHQAVAIRVQADRAIFLNCRFEGYQDTLYA	419
<i>A. thaliana</i>	384	AQGEFMAQSMGFRNTAGSEGHQAVAIRVQSDRSIFLNCRFEGYQDTLYA	433
<i>Z. mays</i>	334	VEASGFICKNMGFHNTAGAEHRHQAVALRVQGDLAIFYNCRFADFQDTLYV	383
DEG6-1	160	MAKRQFYRSCVITGTVDIFGDSASVFNCLMVIRRPDNLQQNIVLAHGR	209
<i>S. gilgiana</i>	420	QTHRQFYRSCVITGTVDIFGDATSVFNCLITVRKPLENQQNIVTAQGR	469
<i>A. thaliana</i>	434	YTHRQYRSCVIVGTIDIFGDAAAIFQNCNIFIRKGLPGQKNTVTAQGR	483
<i>Z. mays</i>	384	QPRRQFFRNCVVSQGTIDIFGNSAAVFQNCNIIITRRPMDNQQNSVTAHGP	433
DEG6-1	210	VDRHETTGFVLHKRIIGDEKLLPVKNKIRSYLGRPWKEFYARHVIMETEI	259
<i>S. gilgiana</i>	470	IDGHETTGI VLQSCRIE PDKDLVPVKNKIRSYLGRPWKEFSRTVIMDSTI	519
<i>A. thaliana</i>	484	VDKFQTTGFVVHNCIAANEDLKPVKEEYKSYLGRPWKNYSRITIMESKI	533
<i>Z. mays</i>	434	TDPNMKSLVITQNCRLVPDQKLFPPDRFKIPSYLGRPWKEFSRLVIMESTI	483
DEG6-1	260	SDVIDPEGYPWEG-DFGLNTLFYGEYNNTPGAKFDGRVRWKGVRKLKR	308
<i>S. gilgiana</i>	520	GDFIHPGGWLPWQG-DFGLKTLYYAEYSNKGGAQTNARIKWPYHIIKK	568
<i>A. thaliana</i>	534	ENVIDPVGWLRWQETDFAIDTLYYAEYNNKGSSTTSRVKWPQGFVINK	583
<i>Z. mays</i>	484	ADFVKPEGYPWNG-DFALKTLYYAEYNNRGPAGTSKRNVNWPGFHVIGR	532
DEG6-1	308	-SAPRFTVADFIQGTIEWINNESGGVAIPVRYGLYN-	342
<i>S. gilgiana</i>	569	EEAMKFTIENFYQG-DWISASGS----PVHLGL---	596
<i>A. thaliana</i>	584	EEALNYTVGPFLOG-DWISASGS----PVKLGLYDA	614
<i>Z. mays</i>	533	KEAEPFTAGPFI DGAMWLKYTGA----PHILGFKF-	563

Figure 2. Amino acid alignment of clone DEG6-1 with other pectin methylesterases. The predicted sequences of *S. gilgiana* (GenBank accession no. BAA89480), *Arabidopsis thaliana* (GenBank accession no. AAP40488) and *Z. mays* (GenBank accession no. CAA73733) were aligned to display homology. Conserved amino acids in grey. The signature domain of PME protein family is underlined. Gaps were automatically inserted to optimize alignment.

DEG9-9	1	-----STRHS-----LHGGVTEMVTE	16
<i>M. domestica</i>	1	MSPDSSREENVYMAKLAEQAERYEEMVEFMEKVAKTVD-	39
<i>L. esculentum</i>	1	---MASPREENVYMAKLAEQAERYEEMVEFMEKVVAADG	37
<i>S. tuberosum</i>	1	---MASPREENVYMAKLAEQAERYEEMVEFMEKVVAADG	37
<i>M. crystallinum</i>	1	MS-SESSREENVYMAKLAEQAERYEEMVEFMEKVAKMTD-	38
DEG9-9	17	<u>NEELSI EERNLLSVAYKNVIAERRASWRIVSSIEQKEESR</u>	56
<i>M. domestica</i>	40	<u>VEELTVEERNLLSVAYKNVIGARRASWRIISSIEQKEESR</u>	79
<i>L. esculentum</i>	38	<u>AEELTVEERNLLSVAYKNVIGARRASWRIISSIEQKEESR</u>	77
<i>S. tuberosum</i>	38	<u>AEELTVEERNLLSVAYKNVIGARRASWRIISSIEQKEESR</u>	77
<i>M. crystallinum</i>	39	<u>TEELSVEERNLLSVAYKNVIGARRASWRIISSIEQKEESR</u>	78
DEG9-9	57	<u>GNDDHLAAI KEFRAKIESELTSICGRILKLVQSHLLPSAL</u>	96
<i>M. domestica</i>	80	<u>GNEDHVAI IKEYRSKIELELSKICDGLNLLSHLIPSA</u>	119
<i>L. esculentum</i>	78	<u>GNEDHVAS IKEYRSKIESELTSICNGILKLLDSKLGSA</u>	117
<i>S. tuberosum</i>	78	<u>GNEDHVAS IKEYRSKIESELTSICNGILKLLDSKLGSA</u>	117
<i>M. crystallinum</i>	79	<u>GNEDHVSTIKEYRGKIELELSKICDGLNLLSHLIPSA</u>	118
DEG9-9	97	<u>SDESKVFF LKMKGDYHRYLAEFKGTGERKDAADDTLSAYA</u>	136
<i>M. domestica</i>	120	<u>SAESKVFFYLKMKGDYHRYLAEFKGTGERKEAAESTLLAYK</u>	159
<i>L. esculentum</i>	118	<u>TGDSKVFFYLKMKGDYHRYLAEFKGTGAERKEAAENTLSAYK</u>	157
<i>S. tuberosum</i>	118	<u>TGDSKVFFYLKMKGDYHRYLAEFKGTGAERKEAAENTLSAYK</u>	157
<i>M. crystallinum</i>	119	<u>TAESKVFFYLKMKGDYHRYLAEFKGTGAERKEAAENTLLAYK</u>	158
DEG9-9	137	<u>FAQDIAMAALAP IHP IRLGLALNFSVFYIEILNSPDRACD</u>	176
<i>M. domestica</i>	160	<u>SAQDIALAELAP THP IRLGLALNFSVFYIEILNSPDRACN</u>	199
<i>L. esculentum</i>	158	<u>AAQDIANAELAP THP IRLGLALNFSVFYIEILNSPDRACN</u>	197
<i>S. tuberosum</i>	158	<u>AAQDIANAELAP THP IRLGLALNFSVFYIEILNSPDRACN</u>	197
<i>M. crystallinum</i>	159	<u>SAQDIALAELAP THP IRLGLALNFSVFYIEILNSPDRACN</u>	198
DEG9-9	177	<u>LAKQAFDDAIAELDSLGEESYKDSTLIMQLLRNLTWTS</u>	216
<i>M. domestica</i>	200	<u>LAKQAFDEAISELDTLGEESYKDSTLIMQLLRNLTWTS</u>	239
<i>L. esculentum</i>	198	<u>LAKQAFDEAIAELDTLGEESYKDSTLIMQLLRNLTWTS</u>	237
<i>S. tuberosum</i>	198	<u>LAKQAFDEAIAELDTLGEESYKDSTLIMQLLRNLTWTS</u>	237
<i>M. crystallinum</i>	199	<u>LAKQAFDEAISELDTLGEESYKDSTLIMQLLRNLTWTS</u>	238
DEG9-9	217	DVLD---DTIEEEASRYEEDWD----	235
<i>M. domestica</i>	240	DITDDAGDEIKEAS-KRESSE--TQQ	262
<i>L. esculentum</i>	238	DMQDDGTDEIKEATPKPDDNE-----	258
<i>S. tuberosum</i>	238	DMQDDGTDEIKEAAPKPDDNE-----	258
<i>M. crystallinum</i>	239	DNAEEGGDEIKEAAAKRESGEEKPQQ	264

Figure 3. Amino acid alignment of clone DEG9-9 with closely linked 14-3-3 proteins. The predicted sequences of *Malus x domestica* (GenBank accession no. AAV50005), *L. esculentum* (GenBank accession no. P93211), *S. tuberosum* (GenBank accession no. P93784) and *M. crystallinum* (GenBank accession no. P93259) were aligned to display homology. Conserved amino acids in grey. The conserved 14-3-3 domain is underlined. Gaps were automatically inserted to optimize alignment.

co (AAR01523) were differentially regulated during pollen development and following heat stress (Volkov et al., 2005). LMW HSPs were also found to be differentially regulated during seed development in *Triticum aestivum* and *Pisum sativum* (Helm and Abernethy, 1990; Helm et al., 1991). Hence, DEG3-8 may be involved in the pollen or seed development in *D. crumenatum*.

DEG6-1 was strongly expressed in the column and BLAST analysis showed similarity to a pectinesterase domain from the PME gene of *D. crumenatum*. The PME is known to be a plant cell wall associated protein that catalyzes the deesterification of galactosyluronate methyl esters of pectins, releasing protons and methanol

(Frenkel et al., 1998). PMEs affect the pH and ionic balances within cell walls (Grignon and Sentenac, 1991) and consequently, the activity of a wide range of hydrolytic enzymes and the interactions between structural cell wall components (Pressey, 1984; Varner and Lin, 1989).

Although the biochemical mode of action of PME is well known, it had been difficult to demonstrate the exact role for PME in plant physiology. However this enzyme has been associated with pollen germination and/or tube growth (Mu et al., 1994), fruit softening during postharvest fruit ripening (Zeng et al., 1996) as well as plant defence (Chamberland et al., 1991; Wietholter et al., 2003).

The PME protein was reported to exhibit male flower-

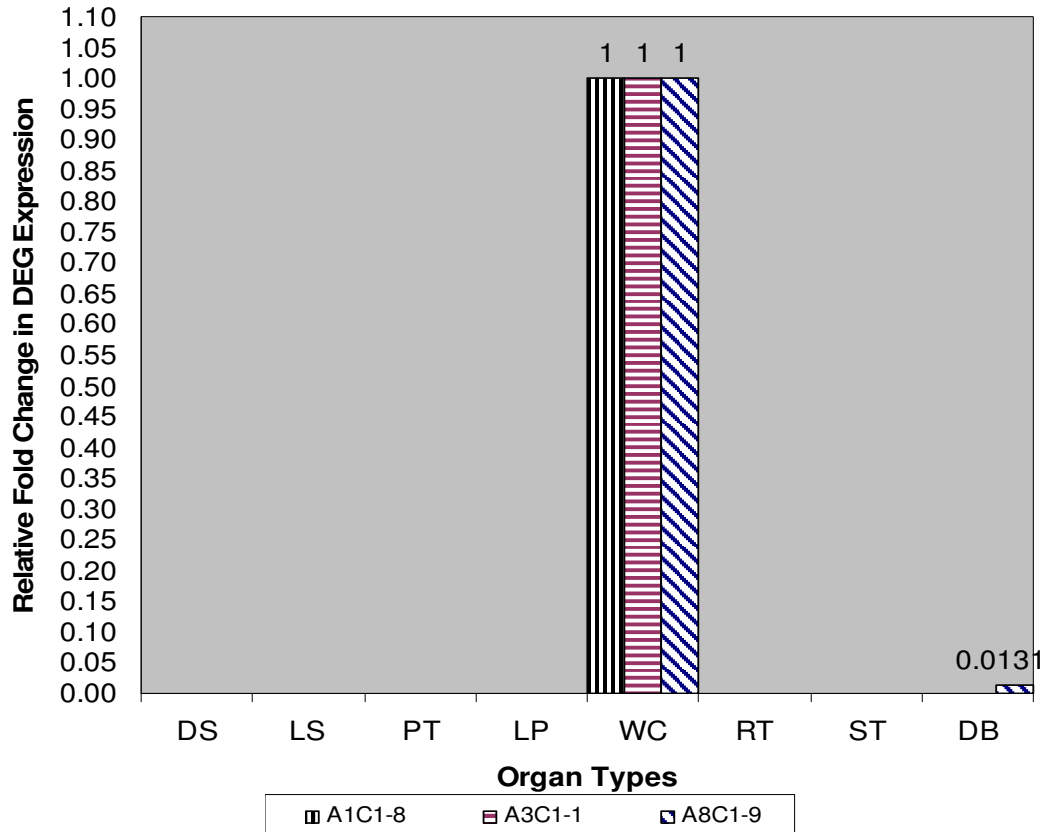


Figure 4. Relative expression level of DEGs in various orchid organs quantified by real-time RT-PCR using the standard curve method. Total RNAs from each organ were reverse transcribed and approximately 150ng cDNAs were subjected to real-time RT-PCR using clone specific primers. All PCRs were conducted in triplicate and normalized to Cyclophilin. Each of the normalized relative values were further divided by the value of the calibrator (column) and the relative expression level is presented as an n-fold expression differences compared to the calibrator organ, column. DS, dorsal sepal; LS, lateral sepal; PT, petal; LP, lip; WC, column; RT, root; ST, stem; DB, developing bud.

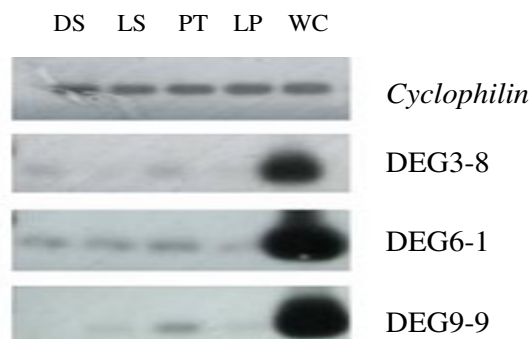


Plate 2. Semi-quantitative RT-PCR analysis via Southern hybridization with biotin-labelled probes. DS, dorsal sepal; LS, lateral sepal; PT, petal; LP, lip; WC, column.

specific expression in willow (*S. gilgiana*) (BAA89480) (Futamura et al., 2000) while a maize PME-like gene, the ZmC5 gene (CAA73733), was also reported by Hussey

(1998) to be specifically expressed in pollen. DEG6-1 may potentially encode partial cDNA sequence of the PME gene from *D. crumenatum*, which has specific expression in the column.

DEG9-9 was also strongly expressed in the column and BLAST analysis showed similarity to the 14-3-3 protein. The 14-3-3 proteins, of approximately 30kDa, exist as homo and heterodimeric molecules and interact with various other proteins involved in signal transduction pathways. The name '14-3-3' actually denotes the elution fraction containing these proteins following DEAE-cellulose chromatography and their migration position after subsequent starch gel electrophoresis. 14-3-3 proteins mainly bind proteins containing phosphothreonine or phosphoserine motifs and have the potential to affect intracellular signalling in three modes, either by direct regulation of the catalytic activity of the bound protein, by regulating interactions between the bound protein and other molecules in the cell by sequestration or modification or by controlling the subcellular localization of the bound ligand (Michael, 2002). Despite the

diverse proteins that have been found to interact with the 14-3-3's, there exist a high degree of sequence identity and conservation between all the 14-3-3 isotypes.

Each 14-3-3 protein sequence can be roughly divided into three sections:

- i) A divergent amino terminus.
- ii) The conserved core region.
- iii) A divergent carboxyl terminus.

The conserved middle core region of the 14-3-3s, forms the main functional domain and encodes an amphipathic groove which acts as a domain for interacting with target proteins. In plant systems, the 14-3-3's were found to be up regulated during apple fruit ripening (AAV50005), functioned as regulatory growth factors that were developmentally regulated in *Brassica napus* (AF342780) as well as regulatory proteins involved in defence response in tomato (Roberts and Bowles, 1999).

Conclusion

Nine differentially expressed genes (DEGs) were detected in the five floral organs of *D. crumenatum* and three DEGs of relatively high expression in the column, were significantly homologous to the small heat shock protein (HSP) differentially regulated during pollen development and heat stress in tobacco (DEG3-8), pectin methylesterase enzyme (PME) which was a male-flower specific gene in *S. gilgiana* (DEG6-1) and the 14-3-3 protein which was differentially expressed and up regulated in *Malus x domestica* (DEG9-9) during fruit ripening.

Generally, the differential expression of the DEGs in the column and other floral organs were distinct, indicating the suppressive role of column DEGs on other floral organ DEGs. Interactions between the other floral DEGs (excluding column DEGs) indicated pattern expression specificity to each floral organ. Following this investigation, further molecular expression investigations are required and essential to develop and establish an orchid floral ontogenic model.

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