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Identification and sequence analysis of cDNA fragments relative to ovary development of *Cymbidium hybridium* after pollination

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Abstract

In this study, seven cDNA fragments of genes, differentially expressed in ovaries after pollination in *Cymbidium hybridium*, were identified and characterized by mRNA differential display reverse transcription polymerase chain reaction (DDRT-PCR). Four (CDD-313, CDD-272, CDD-265, CDD-243) among these seven cDNA fragments showed no significant homology with ESTs or genes in the databases of NCBI; another three (CDD-193, CDD-218, CDD-470) showed significant homologies with sequences encoding components of an ABC-type transporter, a GTPase and 40S ribosomal S3 proteins (RPS3), respectively. The differential expression patterns of them were confirmed by reverse Northern dot blot analysis. More interestingly, CDD-470 appeared to be present and highly expressed in the pollinated ovaries and encoded a new factor of RPS3 participating in cell growth and proliferation. We deduced that this 40s ribosomal S3 like protein was involved in ovary development of orchids.

Additional key words: differential display RT-PCR, floral development, 40S ribosomal S3 protein (RPS3).

Introduction

Floral development in *Arabidopsis* and other plants has been the focus of classic and molecular genetic analysis in recent years (Bowman *et al.* 1989, Coen *et al.* 1990, Schwarz-Sommer *et al.* 1990). The studies of floral homeotic mutants of *Arabidopsis* and *Antirrhinum* led to the proposal of the ABC model of floral development (Coen *et al.* 1991, Weigel *et al.* 1994), which has explained the genetic control of flower morphogenesis. With the development of molecular biology technologies and theories, some floral development genes have been isolated and cloned from different plant species such as gymnosperm and angiosperm (Tandre *et al.* 1998, Theissen *et al*. 2001, Taylor *et al*. 2002, Tsaftaris *et al.* 2005). Through the research on interactions of these genes, rapid progress will be made in further elucidation of the molecular mechanisms involved in the floral development (Weigel *et al.* 1994, Colombo *et al.* 1995). Ovary is one of the most important parts of mature floral organs, so isolation and further functional characterization of genes in ovary will be facilitated in providing new insights into the molecular mechanisms of floral development (Grossniklaus *et al*. 1998).

Cymbidium hybridium is a member of the family *Orchidaceae*, one of the largest and most diverse families of flowering plants. Like other flowering plants (Zik *et al.* 2003), the development of orchid flowers begins with the floral transition and continues with the initiation and formation of floral organs. Being different from the flowers in other flowering plants, the nearly identical shape of sepals and petals as well as the production of a unique large lip (labellum) and a gynostemium or column in orchid flowers make it as a very special plant species in the study of floral development (Yu *et al.* 2000). Furthermore, for other flowering plants, oocytes are not fertilized with powders until ovaries maturity (Bouman *et al.* 1984), whereas ovule development of orchids only starts after successful pollination. So ovary of orchids is an ideal tissue material to study flower ovule initiation and development (Wang *et al.* 1999, Zhu *et al.* 2004). At present, some special genes related to ovary development of orchids have been isolated (Colombo *et al*. 1995, Lu *et al.* 1996, Bui *et al.* 1998, Favaro *et al.* 2003, Ferrario *et al.* 2004). However, limited information is available concerning genes regulating the development of orchid

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Abbreviations: BLAST - basic local alignment search tool; DDRT-PCR - differential display RT-PCR; PAGE - polyacrylamide gel electrophoresis; PCR - polymerase chain reaction; RPS3 - 40s ribosomal S3 proteins; RT-PCR - reverse transcription-PCR.

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ovaries. Therefore, the isolation of more new variant ovary genes in orchids and further studies of their roles in orchid floral development are necessary.

Here, differential display RT-PCR (DDRT-PCR) methods were used to study the changes of gene expression between the non-pollinated ovaries and the pollinated ones in *C. hybridium*, as a step towards the understanding of the molecular mechanism of ovary development in orchids after pollination. Combined with previous horticultural and physiological studies (Goh 1977, 1985), the identification of seven novel genes differentially expressed in ovaries after pollination in this paper will provide a better understanding of the floral development of orchids.

Materials and methods

Plant materials: *Cymbidium hybridium* used in this study was grown in greenhouses under natural light and the temperature was controlled ranging from 23 to 27 °C. Totally 100 flowers were treated while attached to the plant, fifty flowers of which were pollinated after flowering for 20 - 30 d, while the others kept untreated. Following treatments (Wu *et al.* 2004), ovaries collected from pollinated (2 d after pollination) and non-pollinated (the control) flowers of *C. hybridium* were simultaneously dissected, immediately frozen in liquid nitrogen, and stored at -80 °C.

RNA extraction and reverse transcription: Frozen ovaries of the pollinated flowers and the untreated ones were ground in liquid nitrogen and simultaneously extracted using *TRIZOl*® (*BBI*, Markham, Canada), according to the manufacturer's instructions. The possible contaminated DNA was digested by RNase free DNaseI (*Takara*, Shiga, Japan) for 30 min at 37 °C. The RNA was checked for quality and quantity by agarose gel electrophoresis (1.2 % formaldehyde denaturing agarose gel) and fluorimetry (*ND-1000*, *NanoDrop*, Wilmington, USA), respectively.

The total RNA extracted from ten random individual samples was equivalently pooled and subjected to the following reactions. 2 μg total RNA in every reaction were reverse transcribed using 0.5 μg three different reverse primers of M11, M12, M13 (Table 1) respectively and 200 unit M-MLV reverse transcriptase under conditions specified by the enzyme supplier (*Promega*, Madison, USA) in a final volume of 0.01 cm^3.

Differential display analysis: Following reverse transcription, PCR amplifications were performed in 0.025 cm^3 of a reaction mix with 0.001 cm^3 of the reverse-transcribed cDNA, 0.5 μM of each forward (DD-1, 2…26) and reverse primer (M11, M12, M13) (Table 1), 200 μM dNTPs, 1× polymerase buffer and 1 U *Taq* DNA polymerase (*Takara*). PCR amplification reactions were performed in the MG5331 thermal cycler (*Eppendorf*, Hamburg, Germany) using the following programme: after denaturation at 94 °C for 5 min, amplification was performed with 40 cycles of 30 s at 94 °C, 2 min at 40 °C, and 1 min at 72 °C, followed by a final extension at 72 °C for 10 min. Amplified PCR products were separated on a 6 % (m/v) polyacrylamide gel (PAGE). After silver staining (Bassam *et al.* 1991), the gel was dried in room temperature and photographed. A 100-bp ladder was used to estimate the molecular size of fragments. All reactions were performed in triplicate to avoid false-positive results. Only amplification products between 100-bp and 800-bp were considered.

Re-amplification, purification and cloning: The steady differentially displayed bands appearing in all three reactions were excised from PAGE, eluted in 0.03 cm^3 sterile water at 37 °C overnight. For re-amplification of bands, 0.002 cm^3 of three different dilutions (1/10, 1/100 and 1/1000) was used as template in 0.025 cm^3 reaction volume using the same PCR protocol described above. PCR products were separated on a 2 % agarose gel, and cDNA fragments of the appropriate size were extracted from the gel. For cloning, only the re-amplified product from the highest dilution was used, to avoid possible contamination with other minority bands. The target bands were purified by the *QIAEXII* gel extraction kit (*Qiagen*, Valencia, USA), ligated to the *pUCmT*-easy vector (*BBI*) and then transformed into competent cells of *Escherichia coli* DH5α by heat shock (Sambrook *et al.* 1989). Positive clones were identified by PCR methods.

Reverse Northern dot blot analysis: Reverse Northern dot blot was performed as described by Vögeli-Lange *et al.* (1996, 1997) and Li *et al.* (1998) with some modifications. Appropriate amounts of positive clones from differentially displayed bands were dotted and fixed in duplicate sets onto nylon membranes (*Pall*, NY, USA). After the total cDNA from the non-pollinated or the pollinated ovaries had been labeled with digoxygenin (DIG)-11-dUTP by using the random primer DNA labelling system (*Roche*, Basel, Switzerland), reverse Northern hybridization and detection were performed following the instructions of the *Roche DIG* high prime DNA labelling and detection starter kit II. The membranes were then hybridized at 42 °C overnight and were high-stringency washed twice in 2× sodium citrate buffer (SSC) + 0.5 % sodium dodecyl sulphate (SDS) at 42 °C for 30 min, followed twice in 0.5×SSC + 0.5 % SDS at 65 °C for 15 min. β-actin was used as positive control and distilled H_2O was used as negative control.

Sequencing and sequence analysis: Positive clones confirmed by reverse Northern dot blot were sequenced at the *Sangon* (Shanghai, China) using the *PRISM Ready Reaction DyeDeoxy Termination* cycle sequencing kit with an *ABI-3700 DNA Sequencer* (*Applied Biosystems*,

USA). The nucleotide sequences have been deposited in the *GenBank Database* under the accession Nos. DQ159862 - DQ159868. Nucleotide and deduced amino acid sequences derived from positive clones were searched for their similarity using the *BLAST* algorithm (Altschul *et al.* 1997) in GenBank, which was accessed via the NCBI website (http://www.ncbi.nlm.nih.gov/).

Pairwise comparisons and multiple alignments of nucleotide sequences and deduced amino acid sequences were performed using the *Align* program (Myers *et al.* 1988) and *CLUSTAL W* (Thompson *et al.* 1994), with default cost settings for opening and extending gaps. A phylogenetic tree was obtained from this alignment following the neighbour-joining method (Saitou and Nei 1987) with the Poisson correction distance (Nei *et al.* 2002). The reliability of the tree was established by conducting 1000 neighbour-joining bootstrap sampling steps (Felsenstein 1985).

Results

Identification of differentially expressed mRNAs in pollinated ovaries of *Cymbidium hybridium*: Differential display reverse transcription PCR (DDRT-PCR) was used to study the gene differential expression of ovaries before and after pollination. A total of twenty-six 5'-arbitrary primers (DD-1, 2, 3…26) were used. Each of the 5'-primers was paired with one of the three 3'-anchor primers (M11, M12, M13) (Table 1) and used to amplify cDNAs obtained by reverse transcription of total RNA from the control and the pollinated one. To prevent isolation of 'false positives', all amplification experiments were performed on three different dilutions of each cDNA sample. Only cDNA bands whose levels of expression were invariably present in all dilutions were selected for further analysis. In general, after modification of the temperature of annealing, primers concentration and template concentration, 20 - 40 bands of 50 - 1500 bp were displayed in each lane. Only approximately 2 - 10 % of them differed between the two ovaries, of which fragments ranged from 100 to 800 bp were analyzed (Fig. 1).

A total of sixty cDNA bands specifically displayed in pollinated ovaries were identified and extracted from the dried polyacrylamide gels, re-amplified using the same arbitrary primers (Fig. 2). After re-amplification, false-positive fragments could be easily distinguished from true differentially expressed signals (data not shown). In the end, twenty-eight of these sixty cDNA bands were successfully purified and cloned. Since different cDNA species can be contained theoretically within one eluted differentially expressed band (Liang *et al*. 1995), at least ten white colonies obtained by blue-white screening were selected from each clone. The corresponding DNA inserts were tested on reverse Northern dot blot to confirm the true differences of ovaries before and after pollination.

Reverse Northern dot blot analysis: One of the major problems of the differential display technique is the generation of a relatively large number of false positives. To circumvent this problem, positive clone from a single differential display band was further examined by reverse Northern dot blot analysis. From the 28 initial bands, 28 PCR fragments were subjected to reverse Northern dot blot. cDNAs synthesized from RNA isolated from the non-pollinated and the pollinated ovaries were used as probes. Seventeen hybridization signals were generated and detected in the C membrane, eighteen in the P membrane. Of them, the signals of 8 PCR products were found in both the C and the P membrane while seven were only showed in the P membrane (Fig. 3).

Table 1. Primers used in differential display analysis and reverse transcription. * - Primers used to amplify seven candidate genes.

Primer designation	Sequence (5'-3')	Primer designation	Sequence (5'-3')
DD-1	TACAACGAGG	DD-16	GATCACGTAC
DD-2	TGGATTGGTC	DD-17*	GATCTGACAC
DD-3*	CTTTCTACCC	DD-18	GATCTCAGAC
DD-4	TTTTGGCTCC	DD-19*	GATCATAGCC
DD-5	GGAACCAATC	DD-20	GATCAATCGC
DD-6	AAACTCCGTC	DD-21*	GATCTAACCG
DD-7	TCGATACAGG	DD-22	GATCGCATTG
DD-8	TGGTAAAGGG	DD-23	GATCTGACTG
DD-9	TCGGTCATAG	DD-24	GATCATGGTC
DD-10*	GGTACATTGG	DD-25	GATCATAGCG
DD-11	TACCTAAGCG	DD-26	GATCTAAGGC
DD-12	CTGCTTGATG		
DD-13	GTTTTCGCAG	M-11	AAGCTTTTTTTTTTG
DD-14	GATCAAGTCC	M-12*	AAGCTTTTTTTTTTA
DD-15	GATCCAGTAC	M-13*	AAGCTTTTTTTTTTC

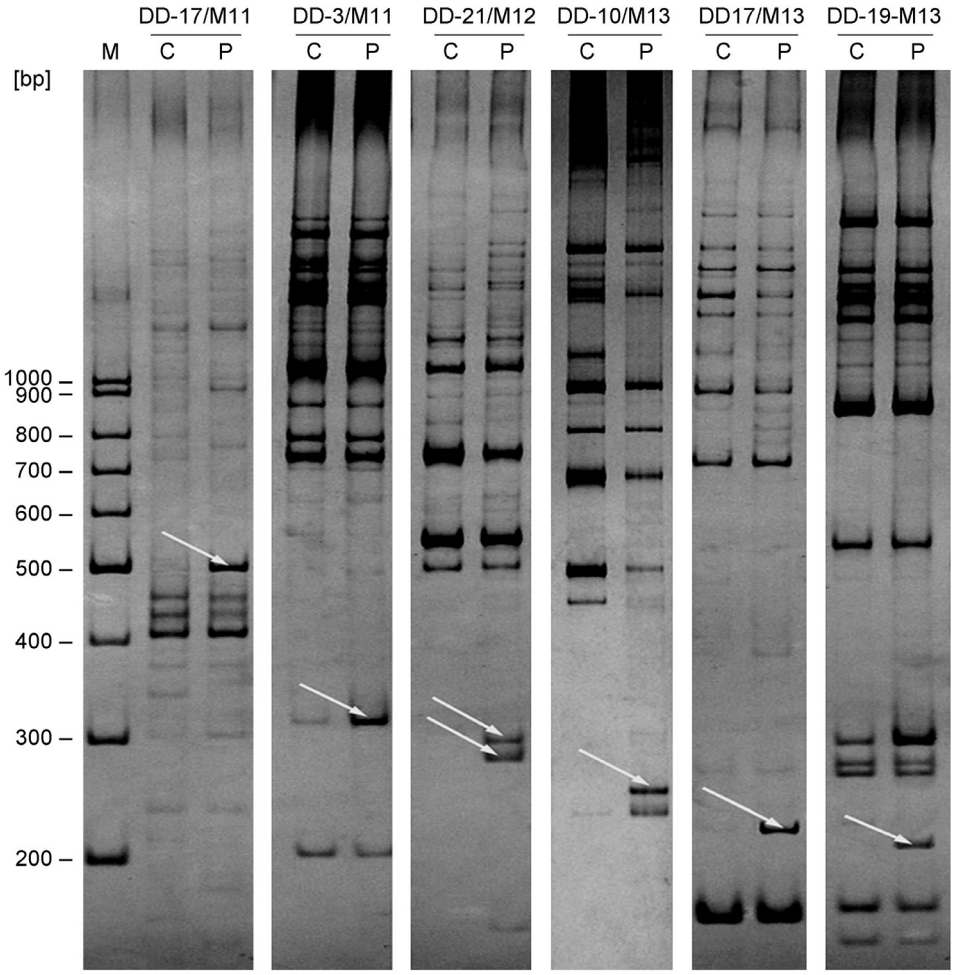

Fig. 1. Parts of representative differential display of mRNA from the control and the pollinated ovaries. Total RNA from control (C) and pollinated (P) samples was reverse-transcribed and amplified with the primer combination as indicated on top of figure. *Arrows* indicate the position of cDNA bands that were recovered from the gels and further analyzed. A 100-bp ladder (M) was used to estimate the molecular size of fragments.

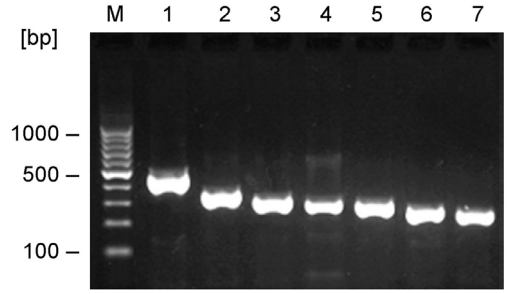

Fig. 2. Parts of re-amplifications of cDNA fragments differentially displayed in pollinated ovaries. The primers used in the re-amplification reactions were the same as shown in Table 1. Lanes 1, 2, 3, 4, 5, 6 and 7 were the re-amplifications of products shown in Fig. 1, respectively. M indicated the molecular mass marker of 100-bp ladder.

Clones showing strong hybridization signals with both probes were classified as false-positives. Finally, these seven only showed in the P membrane were sequenced.

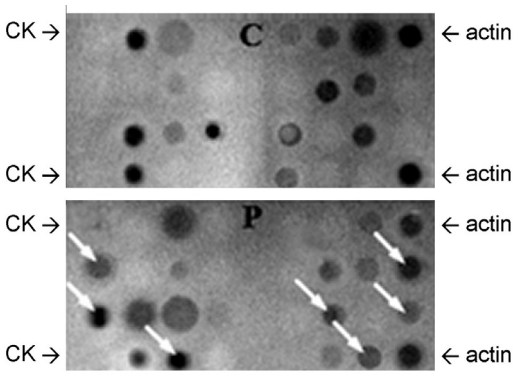

Fig. 3. Reverse Northern dot blot analyses of isolated cDNA fragments. Arrows indicate the position of cDNA bands that were confirmed to be specifically expressed in pollinated ovaries. Actin: positive control from β-actin; CK - negative control from distilled H_2O; P - with pollinated ovary cDNA probes; C - with non-pollinated ovary cDNA probe.

Sequence and phylogenetic analysis: The nucleotide sequences of the differentially expressed cDNA clones specifically obtained from the pollinated ovaries were determined and compared with those published sequences in the *GenBank* databases by *BLAST* searches to identify putative proteins that were encoded by these mRNAs. The nucleotide sequences of these seven cDNA fragments have been deposited in *GenBank*, and their accession numbers along with the results of the *tBLASTX* analysis were summarized in Table 2. Sequence analysis showed that these seven unique cDNA fragments were classified into two categories: unknown (57 %) and signalling/regulation (43 %). Some cDNA fragments may play roles in the induction/regulation of the development of ovaries of *C. hybridium*. For example, CDD-218, CDD-193 and CDD-470 show homology with sequences encoding components of an ABC-type transporter, a GTPase and 40s ribosomal S3 proteins (RPS3), respectively.

On the basis of the deduced amino acid sequence, CDD-470 (DQ159867) appeared to be identical to the corresponding plant ribosomal S3 proteins (RPS3s), dicotyledon *Arabidopsis* (Q42262) and monocotyledon *Oryza sativa* (P49397) (Fig. 4). To determine the evolutionary relationship between CDD-470 and the *RPS3* genes from other species, a phylogenetic tree (Fig. 5) based on analysis of the deduced *RPS3* amino acid sequence of CDD-470 and its homologues from other seven species was constructed. According to the parameters used, the phylogenetic analysis showed that these seven species with CDD-470 had been divided into four large groups, *i.e.*, animals (*Drosophila melanogaster*, *Rattus norvegicus*, *Homo sapiens*), dicotyledons (*Arabidopsis thaliana, Helianthus annuus*), monocotyledons (*Oryza sativa*, CDD-470) and yeast (*Schizosaccharomyces pombe*). It was evident that CDD-470 fell into the clade of plant ribosomal protein S3 genes and it was phylogenetically closely related to the RPS3 of *Oryza sativa* (P49397), which belonged to mono-cotyledons.

Table 2. Sequence analyses of differential display (DD) cDNA fragments in the pollinated ovary *Accession numbers of sequences homologous to DD fragments are in parentheses.

DD products	Accession No.	Primer combination	Length [bp]	Homology*	Identity [%]	Function
CDD-470	DQ159867	DD-17/M11	462	RPS3-*Oryza sativa* (P49397)	89	cell growth proliferation
CDD-313	DQ159862	DD-3/M11	313	no	no	no
CDD-272	DQ159864	DD-21/M12	272	no	no	no
CDD-265	DQ159865	DD-21/M12	265	no	no	no
CDD-243	DQ159863	DD-10/M13	243	no	no	no
CDD-218	DQ159866	DD-17/M13	218	GTPase-*Oryza sativa* (AAX96764)	52	signal transduction
CDD-193	DQ159868	DD-19/M13	193	ABC-transporter-tomato (AAO58969)	62	transport of metabolites

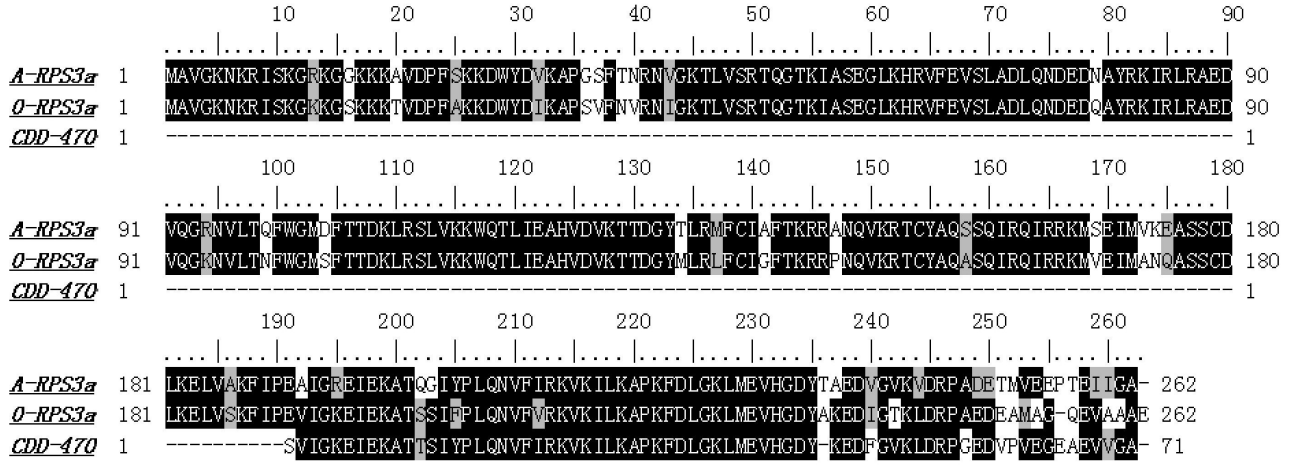

Fig. 4. Alignment of the deduced *C. hybridium RPS3a*, along with the known *RPS3a* from *Arabidopsis thaliana* and *Oryza sativa*, using CLUSTAL W (Thompson *et al*. 1994). Identical amino acids are highlighted by black boxes. GenBank/EMBL/DDJB Nucleotide Sequence Databases, accession numbers: A-RPS3a (Q42262) and O-RPS3a (P49397).

Discussion

The molecular mechanisms involved in floral development are largely unknown. Only recently, by the introduction of molecular methods into plant development, genes involved in floral development have begun to

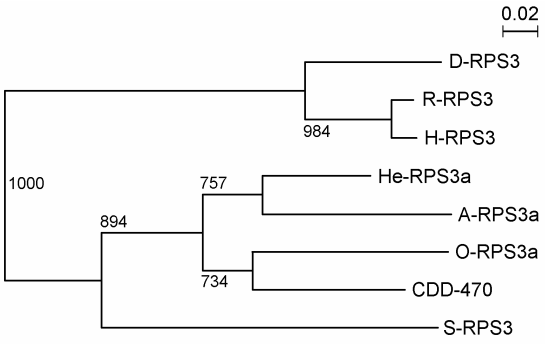

Fig. 5. Phylogenetic tree based on the alignment of the deduced amino acid sequences of *C. hybridium RPS3a* and these seven known RPS3a using the neighbour-joining method (Saitou and Nei 1987). Numbers (%) on the main branches represent bootstrap values (for 1000 iterations). The scale bar in the top right corner displays a distance corresponding to 2 % amino acid substitutions per site. GenBank/EMBL/DDJB Nucleotide Sequence Databases, accession numbers: A-RPS3a (*Arabidopsis thaliana*, Q42262), D-RPS3 (*Drosophila melanogaster*, P55830), S-RPS3 (*Schizosaccharomyces pombe*, Q09781), H-RPS3 (*Homo sapiens,* AAH04981), R-RPS3 (*Rattus norvegicus,* CAA53004), O-RPS3a (*Oryza sativa,* P49397), He-RPS3a (*Helianthus annuus*, P49198) and CDD-470 (DQ159867).

be identified (Tandre *et al.* 1998, Theissen *et al*. 2001, Running *et al*. 2001, Taylor *et al*. 2002). Moreover, the interactions between these genes are poorly understood so far. Similar to most of the flowering plants, the genes associated with the development of flowers in *C. hybridium* are also required to be isolated and identified. For this purpose, DDRT-PCR analysis was performed on the non-pollinated and the pollinated ovaries of *C. hybridium*. In order to avoid minor changes arising from allelic differences between individuals (Ikeda *et al.* 1997) and the large number of false positive bands obtained in differential display reactions, we pooled tissue samples from ten experimental materials and ran all the DDRT-PCR reactions thrice. Only differences between the control and the treatment in all three sets were considered to be positive. Thus, an average of twenty amplification products for each primer combination was obtained (Fig. 1). Sixty products between 100 and 800 bp specifically or highly expressed in the pollinated ovaries were selected for further characterization. In the end, seven cDNA fragments which were confirmed by reverse Northern dot blot to be true differential bands from the pollinated ovaries of *C. hybridium* were isolated and characterized. It suggested that although DDRT-PCR analysis could produce ambiguous results in some cases, considering its rapidity, inexpensiveness and comparative sensitiveness, it was still a powerful technique to study differentially expressed genes.

Among these seven cDNA fragments, four (CDD-313, CDD-272, CDD-265, CDD-243) showed no significant homology with ESTs and genes or strong homology with hypothetical or unknown proteins in the databases; this was probably because these four fragments corresponded to the 3'-untranslated region, where there was typically high sequence variability. And they might be new genes, which would be further confirmed. The other three showed homology with sequences encoding components of an ABC-type transporter, a GTPase and 40S ribosomal S3 proteins, respectively.

CDD-193 (DQ159868), an indication of gene function was given by the three plant proteins (CAB82706 (*Arabidopsis*), AAX95832 (rice) and AAO58969 (tomato)), showed similarity to the ABC-type transporter. ABC-type transporter, ATP-binding cassette transporter system (Choquet *et al.* 2005), is a putative cation transporter with a possible function in signal transduction. Therefore, the putative protein CDD-193 might also act potentially in signal transduction complex. Further studies on structure, function and interaction of the gene product might provide evidences for its relationship to ABC-type transporter and detailed insights into flower development signal transduction mechanisms.

CDD-218, based on the sequence analysis, was inferred to encode a GTPase. Most GTPases have household functions, whereas a few may be required for specialized activities (Borg *et al.* 1997). If the GTPase genes are regulated in mosaic expression patterns, these determinants may have a strong impact on cellular differentiation processes, and hence on tissue and organ development (Terryn *et al*. 1993, Borg *et al*. 1997). Very little is known about these interactions, and we know next to nothing about such events in plant cells. Fortunately, a number of papers and recent reports are beginning to show such researches in plants (Borg *et al.* 1997, Loraine *et al.* 1996, Haizel *et al.* 1995, Yalovsky *et al.* 1996). Our study suggested that CDD-218 might also act potentially in signal transduction, which would provide a new clue to GTPase's functions in flower development.

Of all similar proteins, CDD-470, the predicted gene product from *C. hybridium* yielded the highest similarity with the *Oryza sativa* proteins 89 % (P49397) and amino acid identity with *Arabidopsis* 87 % (Q42262), respectively. The sequence alignment was also performed with *Oryza sativa* and *Arabidopsis* using *CLUSTAL W* (Fig. 4), which implied that the function of the protein might be highly conserved. The strong homology suggested that CDD-470 and plant *RPS3* gene encoded proteins of similar function. Ribosomal protein S3 (RPS3) is one of the ribosomal proteins in the 40S subunit and is located on the surface of the 40S ribosomal subunit. Consistent with its location, several studies

indicated that expression of S3a gene and/or its homologues is required for cell growth and proliferation in various evolutionary distant species (Wilson *et al.* 1994, Yacoub *et al.* 1996, Hegde *et al.* 2001, Sandigursky *et al.* 1997, Deutsch *et al.* 1997, Bommer *et al.* 1991, Kodama *et al.* 1991). A phylogenetic tree demonstrated the relationship between CDD-470 and other *RPS3* genes (Fig. 5). CDD-470 was distinctly closed to *RPS3* from *Oryza sativa* and they were in the same monocot embranchment, which showed that the protein that CDD-470 encoded might have the same functions as the RPS3 from *Oryza sativa*. Furthermore, this fragment was specifically present in the pollinated ovaries, so we deduced that this 40s ribosomal S3 like protein participated in cell growth and proliferation of ovary after pollination, and in turn played certain roles in ovary development of orchids. It was also interesting to indicate that there was the possible functional diversification between the monocot and the dicot during the evolutionary development of flowering. So far, little information is available on the molecular research of ribosomal protein S3 in plants especially in monocotyledonous species. This study provides novel insights into the conservation and diversification of ribosomal protein S3 genes in the floral development of a highly evolved monocotyledonous species.

So far, though these seven cDNA fragments isolated in our experiment are all required to further elucidate, there is no doubt that they are for sure involved in ovary development in *C. hybridium*. These results will make for better understanding of the molecular mechanisms of floral development in *C. hybridium*.

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