Transcription analysis of peloric mutants of *Phalaenopsis* orchids derived from tissue culture

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ABSTRACT

Tissue culture has been widely used for mass propagation of *Phalaenopsis*. However, somaclonal variation occurred during micropropagation process posed a severe problem by affecting product quality. In this study, wild type and peloric flower buds of *Phalaenopsis* hybrids derived from flower stalk nodal culture were used for cDNA-RAPD and cDNA suppression subtractive hybridization analyses in order to study their genetic difference in terms of expressed sequence tags. A total of 209 ESTs from normal flower buds and 230 from mutants were sequenced. These ESTs sequences can be grouped into several functional categories involved in different cellular processes including metabolism, signal transduction, transcription, cell growth and division, protein synthesis, and protein localization, and into a subcategory of proteins with unknown function. Cymbidium mosaic virus transcript was surprisingly found expressed frequently in the peloric mutant of *P*. Little Mary. Real-time RT-PCR analysis on selected ESTs showed that in mutant flower buds, a *bZIP* transcription factor (TGA1a-like protein) was down-regulated, while up-regulated genes include auxin-regulated protein kinase, cyclophilin, and *TCP*-like genes. A retroelement clone was also preferentially expressed in the peloric mutant flowers. On the other hand, ESTs involved in DNA methylation, chromatin remodeling and post-transcriptional regulation, such as DNA methyltransferase, histone acetyltransferase, ERECTA, and DEAD/DEAH RNA helicase, were enriched in normal flower buds than the mutants. The enriched transcripts in the wild type indicate the down regulation of these transcripts in the mutants, and vice versa. The potential roles of the analyzed transcripts in the development of *Phalaenopsis* flowers are discussed.

Keywords: *Phalaenopsis*, tissue culture, peloric mutant, cDNA-RAPD, suppression subtractive hybridization.

INTRODUCTION

Orchid production has become a world-wide business important in floricultural industry [1]. In subtropical and temperate areas, phalaenopsis becomes the most important for orchid production, due to its showy, long-lasting flowers and a large selection of flower colors.

New clones of orchid hybrids are selected annually by different breeding programs and mass propagated through tissue culture using either meristem or inflorescence tip and nodes as starting materials [2-5]. In commercial laboratories, two methods are used to massively produce an elite orchid clone. Shoot multiplication from pre-existed nodal buds or meristems was adopted by many companies. The drawback of the method is the low speed of proliferation of multiple shoots for mass production. To overcome this, others used an alternative approach by inducing protocorm-like bodies (PLBs) from shoot tips or leaves and then further propagating for secondary or tertiary PLBs to obtain a large quantity [2, 3, 6-8]. The PLBs will then differentiate into plantlets in later stage of micropropagation.

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Abbreviations: EST (Expressed sequence tag); cDNA-RAPD (Randomly amplified polymorphic cDNAs); SSH (Suppression subtractive hybridization).

However there are unpredictable mutations or somaclonal variation occurred during the process of multiplication. Some hybrids are more amenable than others to somaclonal variation. The percentage of the variations ranges from 0-100% depending on varieties, with an average of 10% among phalaenopsis hybrids [9]. Ploidy level change was not detected among tested hybrids from tissue culture when analyzed by flow cytometry [9]. However, 2,4-D was reported to affect ploidy levels in suspension cultures of *Doritaenopsis* [10]. Peloric flower as well as flower color mutants occurred in many orchid hybrids through tissue culture process [11, 12].

The cause of somaclonal variation in higher plants has been reported during different biochemical and molecular events, including changes in DNA methylation pattern, activation of transposable elements or retroelements, and chromosome remodeling [13-19]. Rice retrotransposons could be activated during the process of tissue culture [14]. Activation of some retrotransposons has been found linked to chemical and physical causes, and biotic stresses, such as wounding and pathogen infection [17, 20]. Molecular markers have been exploited for the detection of somaclonal variation, including RAPD [21, 22], Methylation sensitive RFLP [18, 23, 24], and microsatellite sequence variation [25]. In tissue culture derived plantlets of oil palms, reduced level of DNA methylation in general has been observed [18]. Somaclonal variation has also been reported in phalaenopsis by RAPD analysis on regenerated plants, which showed morphological and physiological changes in the flowers [21]. The microsatellite instability could be induced by mutation in mismatch repair genes and by pathogen infection in the inflorescence [25-27].

Epigenetic changes in orchids have not been studied in depth so far. Due to limited information of classical genetic map and genomic sequences for phalaenopsis orchid, conventional molecular biological approach becomes the major tool for analysis of orchid development and developmental regulation. The variable genome sizes of *Phalaenopsis* among different species [28-30] make the genetic analysis more complicated. Mutants obtained from different methods, such as chemical mutagenesis, T-DNA insertion, and *in vitro* culture, provide opportunities to pursue developmental process of orchids. In this report, we described transcript profiling of a phalaenopsis peloric flower mutant derived from tissue culture, using randomly amplified polymorphic cDNAs (cDNA-RAPD) and suppression subtractive hybridization (SSH) methods. Expression levels of selected cDNA clones were then compared to the wild type as well as peloric and semi-peloric mutants using real-time RT-PCR to achieve informative understanding of peloric mutants from transcripts level.

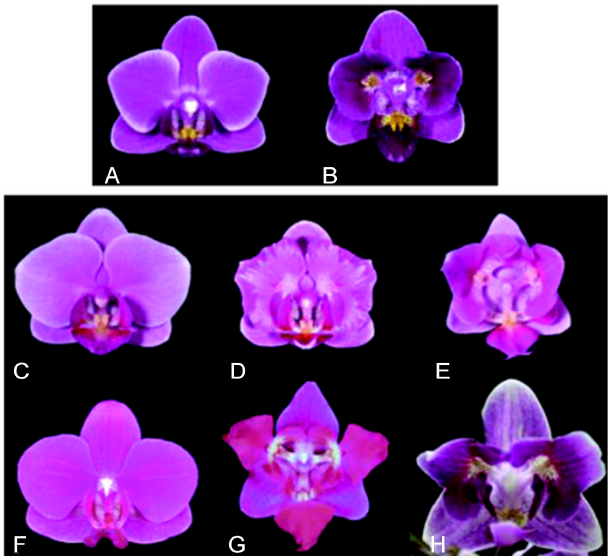

Fig. 1 Morphology of *Phalaenopsis* flowers used in the experiments. **(A)** Wildtype flower of *P*. Zuma's Pixie '#1', **(B)** Semi-peloric flower of *P*. Zuma's Pixie '#1'. **(C)** Wildtype flower of *P*. Little Mary, **(D)** Semi-peloric flower of *P*. Little Mary, **(E)** Peloric flower of *P*. Little Mary. **(F)** Wildtype flower of *Doritaenopsis* Minho Diamond 'F607'. **(G)** Peloric flower of *Dtps.* Minho Diamond 'F607', **(H)** Peloric flower of *P*. hybrid D.

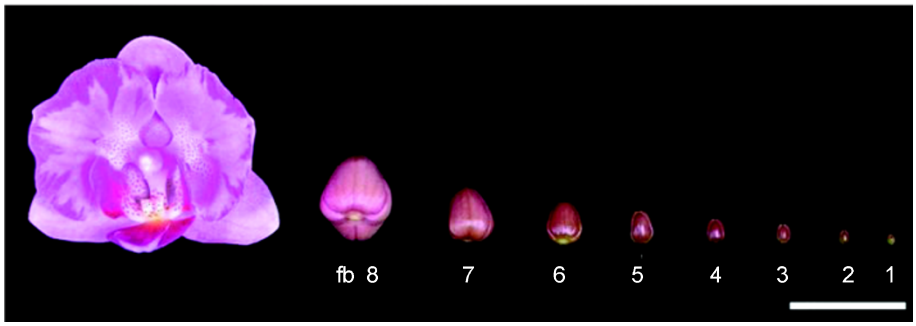

Fig. 2 Developmental stages of flower buds of ***P***. Little Mary. (fb1-fb8) (bar = 2 cm)

MATERIALS AND METHODS

Plant materials and RNA isolation

For cDNA-RAPD analysis, four different sets of *Phalaenopsis* plants were used, including wildtype and semi-peloric flowers and flower buds of *P*. Zuma's Pixie (Obtained from Taida Orchids, Changhwa, Taiwan), *P*. Little Mary 'F535' and *Doritaenopsis* Minho Diamond 'F607' (obtained from Sogo Orchids, Pingtung, Taiwan). For cDNA subtraction, *P*. Little Mary 'F535' and an unknown hybrid (designated as *P*. D) were used (Fig. 1). For all experiments, flower buds younger than stage 4 (Fig. 2) were harvested and frozen in the liquid nitrogen then stored at -80ºC until use.

Total RNAs were isolated and purified using the TRIZOL reagents according to the manufacturer's instructions (Invitrogen). RNA precipitates were resuspended in DEPC-treated sterile water and precipitated with LiCl (a final concentration of 2M). The supernatant was treated with RNase-free DNase (Promega) at 37ºC for 30 min to remove residual genomic DNAs. Purified RNAs were quantified using a spectrophotometer (Hitachi U2000) and quality checked by agarose electrophoresis.

cDNA synthesis, RAPD, and cloning of DNA

Double stranded cDNAs were synthesized from 1 μg total RNAs using the SMART PCR cDNA Synthesis Kit following the manufacturer's instructions (Clontech). For RAPD analysis, the random decamer primers were obtained from Operon. The reaction mixtures contain 200 μg cDNA, 1×PCR buffer, dNTPs, 2 μM random primer, and 1U of Taq DNA polymerase (Takara) in a total volume of 20 μl. The PCR condition was as follow: 94ºC for 5 min, then 35 cycles of 94ºC 30 s, 38ºC 30 s, 72ºC 1 min, and finally an extension of 7 min at 72ºC. The amplified DNA products were separated on 2% agarose gel.

Differentially amplified DNA bands were purified and ligated into pGEM-T Easy vector (Promega). The positive inserts were checked by PCR using universal primers T7 and SP6. The cloned DNA fragments were sequenced with the ABI PRISM 3100 sequencer.

Suppression subtractive hybridization and DNA sequencing

To compare gene expression in flower buds of the peloric mutants and wild type of *P.* Little Mary, SSH was conducted as described previously [31]. For comparison of differential gene expression in flowers and leaves, the peloric unknown hybrid *P*. D with similar flower color and size was used. Poly A^+ RNAs were isolated from 75 μg total RNAs using the Oligo $(dT)_{25}$ containing Dynabeads following manufacturer's instruction (Dynal Biotech). Equal amounts of poly A^+ RNAs (2 μg) from flower buds and leaves were converted into double stranded cDNAs using the SMART PCR cDNA Synthesis Kit (Clontech).

Two subtracted libraries were constructed by using the PCR-Select cDNA Subtraction kit (Clontech). In both libraries, the wild type flower buds of *P*. Little Mary were used as driver and peloric flower buds as tester, and vice versa. A third subtracted library was constructed to compare the transcripts in flower buds and leaves, the *P*. D hybrid leaves were used as driver and peloric flower buds as tester. The subtracted clones were purified and cloned into pGEM-T Easy vector and positive inserts were sequenced.

Tab. 1 Primers used for real-time PCR analysis

Primer name	Sequence (5'→3')	Length (mer)
Endogenous control[a]		
ACTIN4-1	TTGTGAGCAACTGGGATGACAT	22
ACTIN4-2	GCCACGCGAAGTTCATTGT	19
Target genes		
D20-1	CCGTCTGCAATTTTTTCCTAATG	23
D20-2	CCTGCCTTTCCTTTAGC	20
OPT05M-50-1	GTTCAAGACAATGACGACGTTTATG	25
OPT05M-50-2	GTGGATGAAAAGCAAGGAAAGTG	23
ZOPAA01M-2(72)-1	CATGTCGTCACCGGCGTATAC	21
ZOPAA01M-2(72)-2	ACAACGCCACCAGATACCATATTAA	25
ZOPB10M-60-2(1)-1	TCTCCCTGTTAGAAGTTCAAGAAGTACTAC	30
ZOPB10M-60-2(1)-2	GCCATCTCACCACCAAAAGC	20
ZOPAA12M-24(65)-1	GTCCCCAAGACTGCAGAAAACT	22
ZOPAA12M-24(65)-2	TGCGGCCTATACCCATTTCA	20
607FD12-1	GAGAGATCAAGACCAACTAAAACAT	25
607FD12-2	GCTGCTTCCCTTTGTCGATT	20
LOPW12M-4-1(7)-1	CACGGGTGACCTGCTAAATTC	21
LOPW12M-4-1(7)-2	GCTCCTGATCTTTTGCGTTTG	21
LOPW02N-3-2(3)-1	ATAGTCAAAGGATGCTCCTCGAGATA	26
LOPW02N-3-2(3)-2	GATTTGTTTGCTTCCTGGTCATAA	24
LOPW06N-11-3(19)-1	CGCCCGTTGCGAGATCT	17
LOPW06N-11-3(19)-2	CATTAGTATTTAGGCCCGATGGAA	24
LOPW07N-13(29)-1	GGATTTCTGAAGGTCTCGGACAT	23
LOPW07N-13(29)-2	GCCCTGATGGATTTCCTGATT	21

[a]Accession no.: AY134752

Sequence analysis

The cDNA sequences were compared for similarities against GenBank with the BLASTX [32]. The resulted matches were annotated. Classification of annotated sequences was according to the method described in [33].

Real-Time reverse transcriptase PCR assay

Real-time RT-PCR and data analysis were performed in the ABI PRISM 7900 Sequence Detection System using 2×SYBR Green Master Mix to monitor dsDNA synthesis (Applied Biosystems). The gene-specific forward and reverse primers (Tab. 1) were designed based on cloned partial cDNA sequences following the directions of Primer Express 2.0 (Applied Biosystems) and synthesized by Mission Biotechnology (Taipei). Each primer pairs was designed to amplify around 60-80bp length to allow optimized estimation. For controlling the integrity of RNA and normalizing target RNA copy numbers in mutant and wild type flower buds, the housekeeping gene *actin* (PACT4, AY134752) was amplified by real-time RT-PCR to generate a standard curve of *actin* mRNA levels. In order to distinguish the expression levels of individual target genes, their standard curves, used as the calibrators, were established using single stranded cDNAs of the wild type flower buds.

PCR condition was essentially as described in Czechowski *et al.* [34] except that the total reaction volume was 25 µl for each sample and half amount of the 2×SYBR Green (12.5 µl) was added. We found this amount of the dye sufficient for PCR analysis. PCR condition was as follow: 50ºC for 2 min, 95ºC for 10 min, then 40 cycles of 95ºC 15 s, 60ºC 1 min, after that, one more cycle at 95ºC 15 s, 60ºC 15 s and 95ºC 15 s. Standard curve (Ct value against log ng template) for each target and housekeeping gene was established according the guides provided by the ABI PRISM 7900 Sequence Detection System.

RESULTS

Morphology of peloric mutant flowers

Wild type Phalaenopsis flowers possess three petal-like sepals, with one in the top or dorsal sepal, and two lower lateral sepals. There are two lateral petals and a specialized enlarged flamboyant bottom petal, called lip or labellum (Fig. 1A, C, F). In the middle of the flower there is a pistil/stigma fused together with pollinia to form so called column or gynostemium (Fig. 1). In rare cases some seedlings from sexual hybridization may generate lip-like lateral petals. When a selected orchid plant was propagated by flower stalk nodal culture, some clones showed differ-

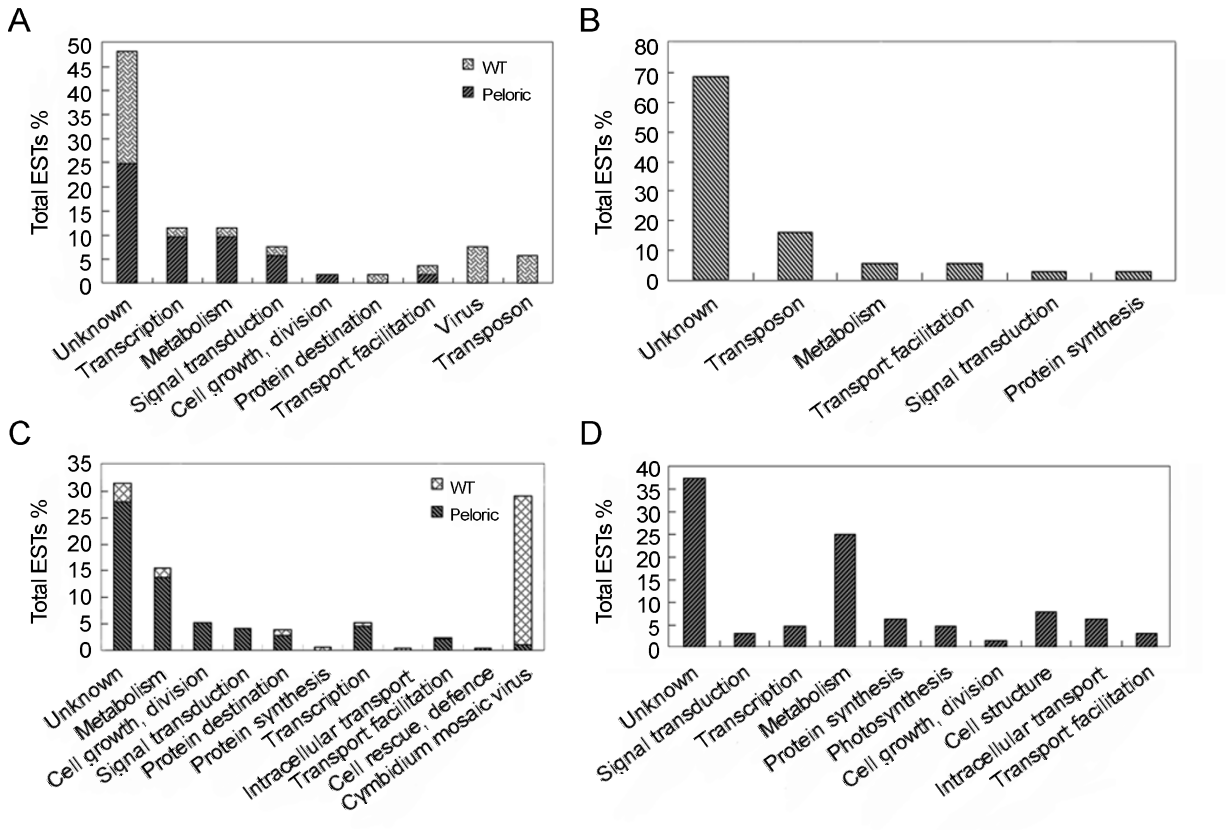

Fig. 3 Functional category of the ESTs in Phalaenopsis flower buds. **(A)** ESTs from wild type (WT) and peloric mutant flower buds by cDNA-RAPD. **(B)** ESTs preferentially enriched in flower buds from cDNA-RAPD flower-to-leaf analysis. **(C)** ESTs enriched in either wild type or peloric mutants of *P.* Little Mary after SSH. **(D)** ESTs enriched in the peloric flower buds of *P.* Little Mary after flower-leaf SSH.

Tab. 2 Homology search of ESTs enriched in wildtype flowers by cDNA-RAPD flower-flower assay

Clone ID	Description[a]	Score (bits)[b]	E value[c]
LAA02N-3-2(3)	RNA polymerase sigma subunit SigE [*Arabidopsis thaliana*]	152	4.00E-36
LOPW02N-3-2(3)	DNA binding protein TGA1a homolog [*Arabidopsis thaliana*]	224	1.00E-57
LOPW02N-3-3(5)	putative arabinosyl transferase [*Mycobacterium leprae*]	33.1	0.72
LOPW06N-11-2(17)	unknown protein [*Arabidopsis thaliana*]	80.5	2.00E-14
LOPW06N-11-3(19)	protein kinase [*Zea mays*]	292	2.00E-78
LOPW06N-11-4(21)	putative protein [*Arabidopsis thaliana*]	89.7	2.00E-17
LOPW07N-13(29)	proline iminopeptidase [*Arabidopsis thaliana*]	224	1.00E-57
LOPW10N-19(39)	putative Rhizobium-induced nodule development associated protein [*Oryza sativa*]	52	1.00E-06
LOPX01N-41(47)	ABC transporter family protein [*Arabidopsis thaliana*]	33.9	1.9
LOPX03N-45-2(51)	TCTR2 protein [*Lycopersicon esculentum*]	72	3.00E-12
LOPX11N-61(57)	putative serine carboxypeptidase I[*Arabidopsis thaliana*]	48.1	6.00E-09
LOPX13N-65(60)	hypothetical protein [*Pyrococcus horikoshii*]	32.7	0.89
OPF08N-15(9)	putative SEC1 family transport protein [*Arabidopsis thaliana*]	116	3.00E-25
OPW04N-7	putative amidase [*Arabidopsis thaliana*]	105	2.00E-22
OPW06N-11	agCP14211 [*Anopheles gambiae* str. PEST]	32.3	0.002
OPW20N-39	putative cellulosomal anchoring protein [*Ruminococcus flavefaciens*]	32.7	3.4
OPX13N-65(2)	unknown protein [*Arabidopsis thaliana*]	98.6	1.00E-20
OPX13N-66	anthocyanidin synthase [*Torenia fournieri*]	67.4	3.00E-11
OPY06N-11	centromere protein-like [*Arabidopsis thaliana*]	73.2	6.00E-14
OPY09N-17	putative pollen specific protein SF21 [*Oryza sativa*]	176	2.00E-52

ent degrees of lip-like lateral petals, due to somaclonal variation. Mild change caused the lateral petals bulge slightly near the center, which was named semi-peloric flowers (Fig. 1D). In the severe case of somaclonal variation, enlarged central bulge of the lateral petals led the transition to lip-like, which was named peloric flowers (Fig. 1 B, E, G, H). The degree of the peloric flower formation varies in different *Phalaenopsis* hybrids as shown in Fig. 1. In severe cases, peloric flowers lost their pollinia (Fig. 1E). When the peloric mutant was re-propagated by tissue culture, some clones may revert back to the wild type flower morphology, an indication of epigenetic changes. Since peloric or semi-peloric mutants share the same genetic background with the wild type of the same variety, we took the advantage to investigate the peloric mutants on molecular level by using cDNA-RAPD and suppression subtractive hybridization techniques.

Differentially expressed ESTs by cDNA-RAPD

Two hundred random primers obtained from Operon (Operon Technologies, Inc., Alameda, CA), including OPAA01-20, OPB01-20, OPD01-20, OPE01-20, OPF01-20, OPT01-20, OPW01-20, OPX01-20, OPY01-20 and OPZ01-20, were used to amplify cDNAs from peloric and wild type flower buds as well as leaves. No PCR products were observed among 45 primers. Products from other 35 primers showed no difference in amplified DNA fragments. The remaining 120 primers produced a total of 510 differential DNA bands, including 93 from wild type flower buds, 366 from peloric flower buds, and 51 from leaves. Among those differentially expressed cDNAs, 191 bands were randomly picked for further characterization. Other bands were not included in the analysis due to noise background of their DNA sequences. After sequencing, only 90 inserts showed unambiguous sequence reading. The length of these cDNAs ranged from about 200 to 3000 bp. Those commonly expressed cDNAs were excluded for analysis. After searching against the GenBank by BlastX, the gene functions of the cDNA fragments were classified according to Bohnert *et al*. [33]. Among them, 28 cDNA clones were preferentially expressed in the wild type flower buds, and 24 expressed in the peloric mutants (Fig. 3A). Annotation of these ESTs was listed in Tab. 2 and Tab. 3. A majority of the cloned cDNA inserts belongs to the unknown function. Only a small proportion of ESTs encoded proteins involved in transcription, metabolism, signal transduction, etc. (Fig. 3A). The efficiency of the cDNA-RAPD approach seems to be low. Interestingly, transoposons and orchid virus (Cymbidium mosaic virus, CyMV; and Odontoglossum ringspot virus, ORSV) were only detected in the peloric mutants in this experiment (Fig. 3A, Tab. 3).

When transcripts of peloric flower buds were compared to that of the leaves in the same mutant, 38 ESTs were preferentially expressed in the flowers (Fig. 3B). Again, most of the ESTs (68.4%) had no known function when searched against the GenBank (Tab. 4). One of the

Tab. 3 Homology search of ESTs specifically enriched in mutant flowers by cDNA-RAPD flower-flower assay

Clone ID	Description[a]	Score (bits)[b]	E value[c]
LAA10M-20(5)	unknown protein [*Arabidopsis thaliana*]	36.6	0.72
LAA15M-30(47)	TEM-93 ES-beta-lactamase [*Escherichia coli*]	389	e-107
LOPW02M-4-1(7)	Unknown protein [*Arabidopsis thaliana*]	109	2.00E-32
LOPW02M-4-2(9)	zinc finger protein [*Schizosaccharomyces pombe*]	31.6	9.2
LOPW02M-4-3(11)	hypothetical protein 1 - potato retrotransposon Tst1 [*Solanum tuberosum*]	48.5	6.00E-05
LOPW05M-10(13)	En/Spm-like transposon-like protein [*Oryza sativa*]	87.4	3.00E-21
LOPW06M-12-1(23)	ORF2 [*Porcine adenovirus* 4]	33.1	3.2
LOPW06M-12-2(25)	putative reverse transcriptase [*Anopheles gambiae*]	57.8	1.00E-07
LOPW06M-12-3(27)	no hit	–	–
LOPW13M-26(41)	RF4 protein - yeast plasmid	37.7	0.13
LOPW19M-38(46)	putative ABC transporter protein [*Oryza sativa*]	287	2.00E-78
OPT01M-42-2	hypothetical protein~similar to *Arabidopsis thaliana* [*Oryza sativa*]	118	8.00E-26
OPT05M-50	putative WD-repeat protein [*Arabidopsis thaliana*]	189	5.00E-50
OPW01M-2(4)	unknown protein [*Arabidopsis thaliana*]	99	5.00E-20
OPW04M-8-2	putative cytochrome c oxidase subunit [*Arabidopsis thaliana*]	42.4	0.002
OPW07M-14	VMP-like sequence protein VlsE [*Borrelia burgdorferi*]	33.5	2.6
OPW18M-36-2	RNA dependent RNA polymerase [Cymbidium mosaic virus]	293	2.00E-79
OPY06M-12(1)	triple gene block 2 [Cymbidium mosaic virus]	167	4.00E-45
OPY09M-18-2	183 kda protein [Odontoglossum ringspot virus]	140	5.00E-33
ZOPAA01M-2(72)	RNA dependent RNA polymerase [Cymbidium mosaic virus]	571	e-162
ZOPAA01N-1(67)	F6N18.13 [*Arabidopsis thaliana*]	123	6.00E-28
ZOPAA09N-17(85)	hypothetical protein [Plasmodium falciparum 3D7]	34.3	0.45
ZOPAA14N-27(57)	hypothetical protein [Plasmodium falciparum 3D7]	34.3	0.38
ZOPAA17N-33(51)	putative lipoxygenase [*Oryza sativa*]	144	4.00E-34
ZOPB10N-59(27)	p55 [*Theileria orientalis*]	34.3	0.35
ZOPB12N-63-2(29)	no hit	–	–
ZOPB17N-73-1(47)	putative ankyrin [*Arabidopsis thaliana*]	205	8.00E-55
ZOPB18N-75(43)	Putative DEAD/DEAH box RNA helicase protein [*Oryza sativa*]	308	5.00E-83

[a]Sequence description of the highest scoring alignments described by one line summaries.

[b]Scores (bits) assigned to an alignment calculated by summing the scores normalizing with the statistical variables for each position in the alignment.

[c]Expect (E) value describes the statistical significance threshold for reporting matches against database sequences.

ESTs, 607FF03-2, shows homology to *MtN3* of the nodules of *Medicago truncatula* [35] and the *NEC1* of *Petunia hybrida* [36]. *NEC1* was specifically expressed in the nectary of the *Petunia* flowers [36].

Among the identified clones by cDNA-RAPD, most of the ESTs belong to the unknown protein category, partially contributed by unknown genome information of orchid and the incomplete ESTs sequences. Some ESTs were probably representing genes involved in signal transduction, protein-protein interactions and transcription regulation, such as cyclophylin, putative ankyrin [37], auxin-related dual specificity cytosolic kinase, putative DEAD/DEAH box RNA helicase, putative WD-repeat protein, and DNA binding protein TGA1a (Tab. 2, 3). The RNA dependent RNA polymerase of CyMV, as well as several classes of retroelements or transposon has also been detected several times in the peloric flowers (Tab. 3, 4).

Differentially expressed ESTs by SSH

The forward subtractive cloning strategy of SSH (wild type flower buds as driver and peloric mutant as tester) generated 181 clones, and reverse subtractive SSH generated 104 clones (Fig. 3C). The highest percentage in the subtracted clones from both forward and reverse SSH was the unknown EST clones. Among them, 28.1% were from the wild type flower buds, while only 3.5% from the peloric flower buds (Fig. 3C). EST clones responsible for metabolism, cell growth, signal transduction and transcription were present mainly in the wild type (Tab. 5), indicating the biased transcript expression due to either epige-

Tab. 4 Homology search of ESTs enriched in peloric flower buds in cDNA-RAPD flower-leaf assay

Clone ID	Description[a]	Score (bits)[b]	E value[c]
607FD02	Hypothetical protein [*Sulfolobus solfataricus*]	34.7	3.2
607FD03	putative polyprotein [*Zea mays*]	47	4.00E-04
607FD04	putative gag-pol precursor [*Oryza sativa*]	59.3	3.00E-08
607FD05	P0592C06.18 [*Oryza sativa*]	56.6	3.00E-07
607FD05(18)	hypothetical protein 2 - potato transposon Tst1	64.3	2.00E-09
607FD07-1	hypothetical protein FLJ10587 [*Homo sapiens*]	105	9.00E-22
607FD07-2	hypothetical protein [*Neurospora crassa*]	33.5	0.67
607FD08	hypothetical protein [*Chlamydia trachomatis*]	33.9	1.7
607FD11	unknown protein [*Arabidopsis thaliana*]	30	9.1
607FD12	Putative retroelement [*Oryza sativa*]	89.4	8.00E-17
607FD18-2	glyoxalase I, putative (lactoylglutathione lyase) [*Arabidopsis thaliana*]	102	1.00E-21
607FD20-2	extracellular protein [*Lactobacillus plantarum* WCFS1]	40.4	0.037
607FE01	hypothetical protein 3 - potato transposon Tst1	55.1	4.00E-07
607FE03	putative non-LTR retroelement reverse transcriptase [*Arabidopsis thaliana*]	61.6	2.00E-10
607FE06-1	hypothetical protein [*Methanosarcina barkeri*]	35.4	1
607FE06-2	NBS/LRR resistance protein-like protein [*Capsicum annuum*]	44.3	1.00E-09
607FE07	PDR-like ABC transporter [*Oryza sativa*]	437	e-121
607FE09-1	En/Spm-like transposon-like protein [*Oryza sativa*]	116	1.00E-38
607FE09-2	2,3-bisphosphoglycerate-independent phosphoglycerate mutase [Geobacillus stearothermophilus]	35	1
607FE09-3	hypothetical protein [*Oryza sativa*]	43.5	1.00E-05
607FE11	hypothetical protein [*Plasmodium falciparum* 3D7]	35	1
607FF03-2	MtN3-like protein [*Arabidopsis thaliana*]	167	1.00E-40
607FF08	similar to Thyroxine-binding globulin precursor (T4-binding globulin) [*Homo sapiens*]	32	9.3
607FF09	olfactory receptor MOR196-4 [*Mus musculus*]	32	6.4
607FF12	guanosine-3',5'-bis(diphosphate) 3'- pyrophosphohydrolase (spoT) [*Mycoplasma genitalium*]	32.3	6.5
607FF13	putative protein [*Arabidopsis thaliana*]	300	2.00E-80
607FW04	putative amidase [*Arabidopsis thaliana*]	57.4	1.00E-14
607FW06-1	putative non-LTR retroelement reverse transcriptase [Arabidopsis thaliana]	46.2	4.00E-04
607FW06-2	alpha 1B-glycoprotein [*Homo sapiens*]	31.6	3.3
607FW07-1	hypothetical malaria antigen [Plasmodium falciparum 3D7]	37	0.18
607FW07-2	DNaJ domain (prokaryotic heat shock protein) DNJ-5 (dnj-5) [*Caenorhabditis elegans*]	33.9	0.49
607FW09-2	hypothetical protein[*Arabidopsis thaliana*]	230	8.00E-60
607FW11	no hit	–	–
607FW16-2	unnamed protein product [*Homo sapiens*]	35.8	0.15
607FW19-2	threonyl-tRNA synthetase [*Oryza sativa*]	568	e-161
607FX01-1	NIb protein [Potato virus A]	33.5	4.5
607FX01-2	no hit	–	–
607FX06	putative protein [*Arabidopsis thaliana*]	239	2.00E-62
ZOPAA03M-6(81)	[*Anopheles gambiae* str. PEST]	29.6	8.9
ZOPAA11M-22(108)	similar to cyclin-dependent kinase-like 1 (CDC2-related kinase) [*Mus musculus*] [*Rattus norvegicus*]	30.4	5.4
ZOPAA12M-24(65)	cyclophylin-like protein [*Arabidopsis thaliana*]	153	2.00E-36
ZOPB10M-60-2(1)	putative auxin-regulated dual specificity cytosolic kinase [Oryza sativa]	125	1.00E-28

[a]Sequence description of the highest scoring alignments described by one line summaries

[b]Scores (bits) assigned to an alignment calculated by summing the scores normalizing with the statistical variables for each position in the alignment.

[c]Expect (E) value describes the statistical significance threshold for reporting matches against database sequences.

Tab. 5 Annotation of cDNA clones enriched in wildtype flower buds from SSH flower-flower analysis

Clone ID	Description[a]	Score(bits)[b]	E value[c]
NP-1	essential protein for meiotic synapsis [*Oryza sativa* (japonica cultivar-group)]	86.3	9.00E-17
NP-2	unspecific monooxygenase (EC 1.14.14.1) - common tobacco	140	6.00E-49
NP-3	hypothetical protein [*Oryza sativa* (japonica cultivar-group)]	68.2	3.00E-11
NP-4	probable cytochrome P450 monooxygenase - maize (fragment)	129	5.00E-48
NP-5	No significant similarity found.	–	–
NP-6	No significant similarity found.	–	–
NP-7	histone acetyltransferase HAT B [*Arabidopsis thaliana*]	100	3.00E-41
NP-8	P0434B04.3 [*Oryza sativa* (japonica cultivar-group)]	92.8	7.00E-18
NP-9	F2E2.13 [*Arabidopsis thaliana*]	115	3.00E-25
NP-10	3-dehydroquinate synthase (EC 4.2.3.4) – [*Emericella nidulans*]	35	0.24
NP-11	CG3231-PA [*Drosophila melanogaster*]	57.4	1.00E-07
NP-12	hypothetical protein - *Arabidopsis thaliana*	57.4	6.00E-08
NP-13	No hit	–	–
NP-14	zinc-finger protein [*Oryza sativa* (indica cultivar-group)]	57	7.00E-08
NP-15	cytochrome P450 monooxygenase [*Zea mays*]	149	7.00E-54
NP-16	putative condensin complex subunit [*Oryza sativa* (japonica cultivar-group)]	40	0.01
NP-17	AKIN gamma [*Medicago truncatula*]	47.8	5.00E-05
NP-18	unspecific monooxygenase (EC 1.14.14.1) - common tobacco	139	6.00E-49
NP-19	essential protein for meiotic synapsis [*Oryza sativa* (japonica cultivar-group)]	194	1.00E-48
NP-21	unknown protein [*Oryza sativa* (japonica cultivar-group)]	322	2.00E-87
NP-22	essential protein for meiotic synapsis [*Oryza sativa* (japonica cultivar-group)]	174	2.00E-42
NP-23	OSJNBa0033G05.19 [*Oryza sativa* (japonica cultivar-group)]	56.6	1.00E-07
NP-24	putative GTP-binding protein [*Oryza sativa* (japonica cultivar-group)]	178	5.00E-44
NP-25	phragmoplastin 5 - soybean	335	2.00E-91
NP-26	cytochrome P450 family [*Arabidopsis thaliana*]	181	4.00E-45
NP-27	ribosomal protein S8 - Plasmodium falciparum plastid	31.2	4.4
NP-28	AKIN gamma [*Medicago truncatula*]	47.8	5.00E-05
NP-29	essential protein for meiotic synapsis [*Oryza sativa* (japonica cultivar-group)]	86.3	9.00E-17
NP-30	tubulin folding cofactor B [*Arabidopsis thaliana*]	242	2.00E-63
NP-31	transcription factor PCF5 [*Oryza sativa* (japonica cultivar-group)]; TCP	36.6	0.098
NP-32	unknown [*Oryza sativa* (japonica cultivar-group)]	94	9.00E-19
NP-33	RNA dependent RNA polymerase [Cymbidium mosaic virus]	297	4.00E-80
NP-34	unknown [*Oryza sativa* (japonica cultivar-group)]	94	9.00E-19
NP-36	expressed integral membrane protein common family [*Arabidopsis thaliana*]	56	1.00E-07
NP-37	mitochondrial ribosomal protein S2; mitochondrial 28S ribosomal protein S2 [*Homo sapiens*].	30.4	7.8
NP-38	Proteasome subunit alpha type 7 (20S proteasome alpha subunit D) (20S proteasome subunit alpha-4)	143	8.00E-34
NP-39	Proteasome subunit alpha type 7 (20S proteasome alpha subunit D) (20S proteasome subunit alpha-4)	143	8.00E-34
NP-40	expressed integral membrane protein common family [*Arabidopsis thaliana*]	56	1.00E-07
NP-41	26S proteasome non-ATPase regulatory subunit 6 (26S proteasome regulatory particle non-ATPase subunit 7) (OsRPN7)	226	1.00E-58
NP-42	cytosolic glutamine synthetase [*Brassica napus*]	202	1.00E-51
NP-43	No hit	–	–
NP-44	No hit	–	–
NP-45	No hit	–	–
NP-46	catalase [*Prunus persica*]	405	e-112
NP-48	catalase [*Prunus persica*]	407	e-112
NP-49	Proteasome subunit alpha type 7 (20S proteasome alpha subunit D) (20S proteasome subunit alpha-4)	143	8.00E-34
NP-50	No hit	–	–
NP-51	ribosomal protein S7 [*Triticum aestivum*]	53.9	7.00E-07
NP-52	OSJNBb0093G06.14 [*Oryza sativa* (japonica cultivar-group)]	191	2.00E-48

Tab. 5 Annotation of cDNA clones enriched in wildtype flower buds from SSH flower-flower analysis (continued-1)

Clone ID	Description[a]	Score(bits)[b]	E value[c]
NP-54	catalase [*Prunus persica*]	432	e-120
NP-55	betaine-aldehyde dehydrogenase, putative (BADH) [*Arabidopsis thaliana*]	223	1.00E-57
NP-56	catalase [*Prunus persica*]	421	e-116
NP-57	No hit	–	–
NP-58	No hit	–	–
NP-62	hypothetical protein [*Arabidopsis thaliana*]	67	2.00E-10
NP-63	hypothetical protein [*Arabidopsis thaliana*]	67	2.00E-10
NP-64	RISBZ4 [*Oryza sativa*]	157	4.00E-38
NP-66	pyruvate dehydrogenase kinase isoform 1; PDK1 [*Zea mays*]	326	2.00E-98
NP-68	CG1 protein [*Plasmodium falciparum*]	30.4	6.2
NP70	Putative RNA-binding protein [*Oryza sativa*]	98.2	3.00E-20
NP71	putative senescence-associated protein [*Pisum sativum*]	130	4.00E-49
NP72	essential protein for meiotic synapsis [*Oryza sativa*]	172	4.00E-42
NP73	putative condensin complex subunit [*Oryza sativa*]	40	0.01
NP76	pectin methyl esterase [*Solanum tuberosum*]	52	2.00E-06
NP77	ER lumen retaining receptor (HDEL receptor), putative [*Arabidopsis thaliana*]	169	1.00E-41
NP78	cytochrome P450 like_TBP [*Nicotiana tabacum*]	136	7.00E-48
NP79	cytochrome P450 like_TBP [*Nicotiana tabacum*]	136	6.00E-48
NP80	B1139B11.7 [Oryza sativa , phosphoesterase family [*Arabidopsis thaliana*]	130	4.00E-30
NP81	transmembrane protein FT27/PFT27-like [*Arabidopsis thaliana*]	110	5.00E-24
NP82	cytochrome P450 like_TBP [*Nicotiana tabacum*]	136	6.00E-48
NP83	putative senescence-associated protein [*Pisum sativum*]	248	1.00E-64
NP85	AKIN gamma [*Medicago truncatula*]	49.3	2.00E-05
NP87	putative CER1 [*Oryza sativa* (japonica cultivar-group)]	242	3.00E-63
NP88	cyclin family [*Arabidopsis thaliana*]	188	9.00E-47
NP89	putative peptide transporter [*Oryza sativa* (japonica cultivar-group)]	130	5.00E-30
NP90	GTP pyrophosphokinase [*Bacillus anthracis* str. Ames]	45.4	5.00E-04
NP91	No hit	–	–
NP92	Putative RNA-binding protein [*Oryza sativa* (japonica cultivar-group)]	117	4.00E-26
NP93	No hit	–	–
NP94	ER lumen retaining receptor (HDEL receptor), putative [*Arabidopsis thaliana*]	171	5.00E-42
NP95	putative condensin complex subunit [*Oryza sativa* (japonica cultivar-group)]	42.4	0.002
NP96	No hit	–	–
NP97	OSJNBa0027G07.12 [*Oryza sativa* (japonica cultivar-group)]	54.7	6.00E-07
NP98	unspecific monooxygenase (EC 1.14.14.1) - common tobacco	137	2.00E-48
NP99	putative senescence-associated protein [*Pisum sativum*]	244	1.00E-63
NP100	DNA cytosine methyltransferase MET2a [*Zea mays*]	283	3.00E-75
NP101	hypothetical protein [*Plasmodium yoelii yoelii*]	33.1	1.1
NP102	P0701D05.20 [*Oryza sativa* (japonica cultivar-group)]	204	7.00E-52
NP103	expressed protein [*Arabidopsis thaliana*]	168	8.00E-41
NP104	cytochrome P450 family [*Arabidopsis thaliana*]	181	5.00E-45
NP105	unspecific monooxygenase (EC 1.14.14.1) - common tobacco	137	5.00E-48
NP106	OSJNBa0043L24.13 [*Oryza sativa* (japonica cultivar-group)]	86.7	8.00E-17
NP107	putative eukaryotic translation initiation factor 6 [*Oryza sativa* (japonica cultivar-group)]	94.7	4.00E-19
NP108	Putative RNA-binding protein [*Oryza sativa* (japonica cultivar-group)]	100	2.00E-22
NP110	hypothetical protein [*Nicotiana tabacum*]	209	3.00E-53
NP111	essential protein for meiotic synapsis [*Oryza sativa* (japonica cultivar-group)]	191	8.00E-48
NP112	putative condensin complex subunit [*Oryza sativa* (japonica cultivar-group)]	42.4	0.002
NP114	putative PHD-finger protein [*Oryza sativa* (japonica cultivar-group)]	177	2.00E-43
NP115	DYAD [*Arabidopsis thaliana*]	82	3.00E-15
NP116	P0434B04.3 [*Oryza sativa* (japonica cultivar-group)]	90.5	4.00E-17
NP117	ZYG homolog [*Homo sapiens*]	31.2	4.2
NP118	cytosolic class II low molecular weight heat shock protein [*Prunus dulcis*]	141	1.00E-32

Tab. 5 Annotation of cDNA clones enriched in wildtype flower buds from SSH flower-flower analysis (continued-2)

Clone ID	Description[a]	Score(bits)[b]	E value[c]
NP119	putative phenylalanyl-tRNA synthetase alpha chain [*Oryza sativa* (japonica cultivar-group)]	62	2.00E-09
NP120	No hit	–	–
NP121	MNF13.29~unknown protein [*Arabidopsis thaliana*]	67.8	2.00E-10
NP123	Putative RNA-binding protein [*Oryza sativa* (japonica cultivar-group)]	117	3.00E-26
NP124	ER lumen retaining receptor (HDEL receptor), putative [*Arabidopsis thaliana*]	171	5.00E-42
NP126	No hit	–	–
NP129	HAK4 [*Oryza sativa*]	261	3.00E-69
NP131	triple gene block 3 [Cymbidium mosaic virus]	95.5	2.00E-19
NP132	receptor-related protein kinase, ERECTA [*Arabidopsis thaliana*]	176	5.00E-44
NP135	Putative quinone oxidoreductase [*Oryza sativa*]	158	2.00E-38
NP136	zinc-finger protein [*Oryza sativa* (indica cultivar-group)]	55.1	3.00E-07
NP137	unspecific monooxygenase (EC 1.14.14.1) - common tobacco	140	3.00E-49
NP138	NADP dependent malic enzyme [*Oryza sativa* (japonica cultivar-group)]	229	7.00E-60
NP139	ATP citrate lyase [*Arabidopsis thaliana*]	252	7.00E-67
NP140	hypothetical protein [*Plasmodium falciparum* 3D7]	32.3	4.7
NP141	calmodulin 1 [*Brassica oleracea*]	152	2.00E-36
NP143	putative esterase [*Oryza sativa* (japonica cultivar-group)]	155	1.00E-37
NP146	SNF1-related protein kinase [*Hordeum vulgare* subsp. vulgare]	112	1.00E-40
NP147	lipid transfer protein precursor [*Davidia involucrata*]	106	1.00E-22
NP148	unknown protein [*Oryza sativa*]	321	1.00E-92
NP150	unspecific monooxygenase (EC 1.14.14.1) - common tobacco	137	1.00E-47
NP151	F18O14.34 [*Arabidopsis thaliana*]	221	7.00E-57
NP153	essential protein for meiotic synapsis [*Oryza sativa* (japonica cultivar-group)]	52	2.00E-06
NP154	unspecific monooxygenase (EC 1.14.14.1) - common tobacco	139	8.00E-49
NP156	expressed protein [*Arabidopsis thaliana*]	67.8	3.00E-10
NP157	No hit	–	–
NP158	No hit	–	–
NP159	No hit	–	–
NP161	coat protein - Cymbidium mosaic virus	178	4.00E-44
NP162	putative condensin complex subunit [*Oryza sativa* (japonica cultivar-group)]	40	0.01
NP163	OSJNBa0032F06.16 [*Oryza sativa* (japonica cultivar-group)]	161	3.00E-39
NP164	putative senescence-associated protein [*Pisum sativum*]	207	5.00E-54
NP165	essential protein for meiotic synapsis [*Oryza sativa* (japonica cultivar-group)]	244	5.00E-64
NP168	No hit	–	–
NP170	similar to KIAA1205 protein [*Homo sapiens*]	34.7	2.2
NP171	hypothetical protein At2g29670 [imported] - *Arabidopsis thaliana*	136	4.00E-31
NP172	No hit	–	–
NP173	No hit	–	–
NP174	No hit	–	–
NP175	60S ribosomal protein L17 [*Oryza sativa*]	122	1.00E-27
NP176	No hit	–	–
NP177	unspecific monooxygenase (EC 1.14.14.1) - common tobacco	136	7.00E-48
NP178	CG3231-PA [*Drosophila melanogaster*]	57.4	1.00E-07
NP179	putative senescence-associated protein [*Pisum sativum*]	244	1.00E-63
NP180	putative WD repeat protein [*Oryza sativa*]	181	6.00E-45
NP181	putative auxin-repressed protein [*Prunus armeniaca*]	80.5	1.00E-14
NP182	unspecific monooxygenase (EC 1.14.14.1) - common tobacco	136	6.00E-48
NP185	F20D23.20 protein - *Arabidopsis thaliana*	190	1.00E-47
NP186	F20D23.20 protein - *Arabidopsis thaliana*	190	1.00E-47
NP187	putative peptide transporter [*Oryza sativa*]	130	5.00E-30
NP188	unknown [*Arabidopsis thaliana*]	247	2.00E-64
NP189	hypothetical protein [*Oenothera elata* subsp. hookeri]	36.2	0.25
NP191	probable cytochrome P450 monooxygenase - maize (fragment)	129	8.00E-48

Tab. 5 Annotation of cDNA clones enriched in wildtype flower buds from SSH flower-flower analysis (continued-3)

Clone ID	Description[a]	Score(bits)[b]	E value[c]
NP192	putative WD repeat protein [*Oryza sativa* (japonica cultivar-group)]	181	6.00E-45
NP193	ring finger protein (2G644) [*Caenorhabditis elegans*]	32.7	2
NP194	putative RNA-binding protein [*Oryza sativa*]	203	6.00E-52
NP195	metallothionein-like protein type 2 [*Narcissus pseudonarcissus*]	87.8	8.00E-17
NP196	essential protein for meiotic synapsis [*Oryza sativa*]	86.3	2.00E-16
NP197	L-galactono-1,4-lactone dehydrogenase, putative [*Arabidopsis*]	287	2.00E-76
NP198	putative senescence-associated protein [*Pisum sativum*]	251	1.00E-65
NP199	transfactor-like [*Oryza sativa*]	139	2.00E-32
NP200	unknown protein [*Oryza sativa*]	301	4.00E-81
NP201	putative cyclin Ia [*Oryza sativa*]	60.5	2.00E-08
NP203	essential protein for meiotic synapsis [*Oryza sativa*]	86.3	9.00E-17
NP205	histone deacetylase [*Solanum chacoense*]	36.6	0.34
NP206	expressed protein [*Arabidopsis thaliana*]	166	2.00E-40
NP210	unknown protein [*Oryza sativa*]	95.9	1.00E-19
NP212	OSJNBa0058K23.21 [*Oryza sativa*]	130	3.00E-29
NP213	hypothetical protein [*Erwinia amylovora*]	269	4.00E-71
NP215	putative senescence-associated protein [*Pisum sativum*]	255	7.00E-67
NP216	phosphoesterase family [*Arabidopsis thaliana*]	106	4.00E-22
NP217	putative ARP2/3 complex 20 kDa subunit, 3'-partial [*Oryza sativa*]	48.5	9.00E-05
NP219	putative senescence-associated protein [*Pisum sativum*]	243	3.00E-63
NP220	Alpha-S1 casein precursor [*Sus scrofa*]	33.1	1.1
NP221	AKIN gamma [*Medicago truncatula*]	47.8	5.00E-05
NP222	hypothetical protein MG02240.4 [*Magnaporthe grisea* 70-15]	33.1	1.1
NP223	succinate dehydrogenase subunit 3 [*Podophyllum peltatum*]	32.3	2.1
NP224	putative senescence-associated protein [*Pisum sativum*]	245	8.00E-65
NP226	chaperonin, putative [*Arabidopsis thaliana*]	338	8.00E-92

[a]Sequence description of the highest scoring alignments described by one line summaries

[b]Scores (bits) assigned to an alignment calculated by summing the scores normalizing with the statistical variables for each position in the alignment.

[c]Expect (E) value describes the statistical significance threshold for reporting matches against database sequences.

netic changes or virus infection. We observed 80 clones (28.1%) of the ESTs, from peloric flower buds, belonging to the transcripts of the orchid virus, Cymbidium mosaic virus (CyMV). Almost the whole genome of the CyMV, including RNA replicase, RNA-dependent RNA polymerase, movement protein, triple gene blocks, and coat protein, was preferentially enriched in the peloric flower buds after SSH (Tab. 6). In order to overcome the surveillance of host cells by posttranscriptional gene silencing, tombusviruses may preferentially express gene products such as p19 protein to serve as a silencing suppressor so that virus population can be established in host cells [38]. The enriched virus in orchid tissues may also adopt similar strategy as tombusviruses to overcome the surveillance of host cells.

When the peloric flower buds were used as driver, and leaves of the same mutant as tester, 64 EST clones were enriched in the flower buds (Fig. 3D). Again, genes with unknown function and metabolism (such as tocopherol cyclase, polyamine oxidase, arginine decarboxylase, and pectinesterase, Tab. 7) were present in majority (37.5 and 25%, respectively). Two clones (D20, NP-31) containing TCP domain, which may play a role in floral symmetry [39-41], were detected in the peloric flower buds from the flower-to-leaves SSH (Tab. 7), as well as in the wild type from the flower-to-flower SSH (NP-31, Tab. 5), respectively.

Some other ESTs, involved in DNA methylation, chromatin remodeling and post-transcriptional regulation, such as DNA methyltransferase, histone acetyltransferase, ERECTA, and DEAD/DEAH RNA helicase, were preferentially up-expressed in wild type flower buds (Tab. 2, 5) in the wild type-flower/mutant-flower SSH. Thus the ESTs involved in epigenetic regulation are down regulated in the mutants, suggesting abnormal gene silencing in the mutants. Multiple copies of cDNA clones were observed in the SSH experiments, such as unspecific monooxygenase, proteasome subunit alpha type 7, cytochrome P450 like-TBP, RNA dependent RNA polymerase (CyMV), triple gene blocks, etc (Tab. 5, 6). Whether they belong to the real differentially expressed clones or due to background in-

Tab. 6 Annotation of cDNA clones enriched in peloric flower buds from SSH flower-flower analysis

Clone ID	Description[a]	Score(bits)[b]	E value[c]
PN-1	putative nucleic acid binding protein [*Oryza sativa* (japonica cultivar-group)]	75.9	1.00E-13
PN-2	triple gene block 1 [Cymbidium mosaic virus]	161	2.00E-39
PN-4	triple gene block 3 [Cymbidium mosaic virus]	95.5	2.00E-19
PN-6	RNA dependent RNA polymerase [Cymbidium mosaic virus]	398	e-110
PN-7	expressed protein [*Arabidopsis thaliana*]	139	1.00E-32
PN-8	OJ1136_A10.4 [*Oryza sativa* (japonica cultivar-group)]	162	8.00E-40
PN-9	triple gene block 3 [Cymbidium mosaic virus]	95.5	2.00E-19
PN-10	triple gene block 3 [Cymbidium mosaic virus]	95.5	2.00E-19
PN-11	RNA replicase [Cymbidium mosaic virus]	407	e-112
PN-12	RNA dependent RNA polymerase [Cymbidium mosaic virus]	435	e-121
PN-13	RNA dependent RNA polymerase [Cymbidium mosaic virus]	424	e-118
PN-14	triple gene block 3 [Cymbidium mosaic virus]	54.7	4.00E-07
PN-15	triple gene block 1 [Cymbidium mosaic virus]	299	2.00E-80
PN-16	40S ribosomal protein S8	146	7.00E-35
PN-17	unspecific monooxygenase (EC 1.14.14.1) - common tobacco	137	3.00E-48
PN-18	triple gene block 1 [Cymbidium mosaic virus]	424	e-118
PN-19	putative protein [*Arabidopsis thaliana*]	259	2.00E-68
PN-20	triple gene block 1 [Cymbidium mosaic virus]	336	2.00E-91
PN-21	MP1 [Cymbidium mosaic virus]	131	3.00E-30
PN-22	triple gene block 1 [Cymbidium mosaic virus]	161	2.00E-39
PN-23	triple gene block 3 [Cymbidium mosaic virus]	95.5	2.00E-19
PN-24	triple gene block 2 [Cymbidium mosaic virus]	103	1.00E-21
PN-25	triple gene block 1 [Cymbidium mosaic virus]	241	1.00E-85
PN-30	triple gene block 3 [Cymbidium mosaic virus]	95.5	2.00E-19
PN70	putative senescence-associated protein [*Pisum sativum*]	244	2.00E-63
PN71	triple gene block 2 [Cymbidium mosaic virus]	112	2.00E-24
PN72	RNA dependent RNA polymerase [Cymbidium mosaic virus]	293	6.00E-79
PN73	triple gene block 1 [Cymbidium mosaic virus]	334	1.00E-90
PN74	RNA dependent RNA polymerase [Cymbidium mosaic virus]	390	e-107
PN75	RNA dependent RNA polymerase [Cymbidium mosaic virus]	241	3.00E-63
PN76	triple gene block 1 [Cymbidium mosaic virus]	324	8.00E-88
PN77	OSJNBa0070C17.7 [*Oryza sativa*]	63.9	2.00E-21
PN78	triple gene block 1 [Cymbidium mosaic virus]	216	5.00E-55
PN79	RNA dependent RNA polymerase [Cymbidium mosaic virus]	276	5.00E-73
PN80	hypothetical protein wali7 - wheat (fragment)	183	6.00E-46
PN81	RNA dependent RNA polymerase [Cymbidium mosaic virus]	285	2.00E-76
PN82	coat protein [Cymbidium mosaic virus]	261	2.00E-69
PN83	RNA dependent RNA polymerase [Cymbidium mosaic virus]	257	e-111
PN84	MP1 [Cymbidium mosaic virus]	117	4.00E-26
PN85	triple gene block 3 [Cymbidium mosaic virus]	94.4	4.00E-19
PN86	triple gene block 3 [Cymbidium mosaic virus]	103	3.00E-21
PN87	chaperonin 60 alpha subunit [*Canavalia lineata*]	70.9	6.00E-12
PN88	triple gene block 2 [Cymbidium mosaic virus]	125	2.00E-28
PN89	RNA dependent RNA polymerase [Cymbidium mosaic virus]	415	e-115
PN90	triple gene block 3 [Cymbidium mosaic virus]	94.4	4.00E-19
PN91	MP1 [Cymbidium mosaic virus]	131	3.00E-30
PN92	RNA dependent RNA polymerase [Cymbidium mosaic virus]	425	e-118
PN93	triple gene block 1 [Cymbidium mosaic virus]	324	1.00E-87
PN94	triple gene block 1 [Cymbidium mosaic virus]	186	4.00E-46
PN95	unknown protein [*Arabidopsis thaliana*]	64.3	1.00E-09
PN96	putative Rab geranylgeranyl transferase [*Arabidopsis thaliana*]	141	3.00E-33
PN97	triple gene block 3 [Cymbidium mosaic virus]	94.4	4.00E-19
PN98	triple gene block 1 [Cymbidium mosaic virus]	420	e-116
PN99	triple gene block 1 [Cymbidium mosaic virus]	253	2.00E-81

Tab. 6 Annotation of cDNA clones enriched in peloric flower buds from SSH flower-flower analysis (continued-1)

Clone ID	Description[a]	Score(bits)[b]	E value[c]
PN100	triple gene block 1 [Cymbidium mosaic virus]	263	1.00E-69
PN101	triple gene block 1 [Cymbidium mosaic virus]	317	5.00E-88
PN102	coat protein [Cymbidium mosaic virus]	83.6	7.00E-16
PN103	triple gene block 1 [Cymbidium mosaic virus]	289	1.00E-80
PN104	triple gene block 1 [Cymbidium mosaic virus]	292	1.00E-78
PN105	triple gene block 2 [Cymbidium mosaic virus]	110	4.00E-24
PN106	triple gene block 2 [Cymbidium mosaic virus]	110	4.00E-24
PN107	proton pump -related [*Arabidopsis thaliana*]	132	2.00E-30
PN108	calcineurin temperature suppressor Cts1 [Cryptococcus neoformans]	42.2	0.01
PN109	triple gene block 1 [Cymbidium mosaic virus]	322	5.00E-87
PN110	triple gene block 1 [Cymbidium mosaic virus]	343	2.00E-93
PN111	hypothetical protein [*Leptospira interrogans* serovar lai str. 56601]	31.6	3.6
PN112	triple gene block 1 [Cymbidium mosaic virus]	335	3.00E-92
PN113	triple gene block 1 [Cymbidium mosaic virus]	337	1.00E-91
PN114	OSJNBa0091D06.22 [*Oryza sativa* (japonica cultivar-group)]	102	1.00E-20
PN115	triple gene block 1 [Cymbidium mosaic virus	338	4.00E-92
PN116	triple gene block 3 [Cymbidium mosaic virus]	94.4	4.00E-19
PN117	RNA replicase [Cymbidium mosaic virus]	92	2.00E-18
PN118	RNA dependent RNA polymerase [Cymbidium mosaic virus]	405	e-112
PN119	triple gene block 2 [Cymbidium mosaic virus]	112	2.00E-24
PN120	coat protein [Cymbidium mosaic virus]	201	2.00E-51
PN121	RNA dependent RNA polymerase [Cymbidium mosaic virus]	384	e-105
PN122	chaperonin 60 alpha subunit [*Canavalia lineata*]	69.7	1.00E-11
PN123	triple gene block 1 [Cymbidium mosaic virus]	419	e-116
PN124	putative zinc finger protein [*Oryza sativa* (japonica cultivar-group)]	316	4.00E-86
PN125	triple gene block 3 [Cymbidium mosaic virus]	91.3	3.00E-18
PN126	RNA dependent RNA polymerase [Cymbidium mosaic virus]	284	1.00E-75
PN127	putative senescence-associated protein [*Pisum sativum*]	248	4.00E-72
PN129	RNA dependent RNA polymerase [Cymbidium mosaic virus]	287	4.00E-77
PN130	RNA dependent RNA polymerase [Cymbidium mosaic virus]	287	4.00E-77
PN131	RNA dependent RNA polymerase [Cymbidium mosaic virus]	253	e-107
PN132	RNA dependent RNA polymerase [Cymbidium mosaic virus]	317	e-110
PN133	putative coatomer complex subunit [*Arabidopsis thaliana*]	286	2.00E-76
PN134	triple gene block 1 [Cymbidium mosaic virus]	425	e-118
PN135	TPA: hypothetical class II basic helix-loop-helix protein [*Homo sapiens*]	34.3	2.1
PN136	triple gene block 1 [Cymbidium mosaic virus]	342	3.00E-93
PN137	triple gene block 1 [Cymbidium mosaic virus]	302	4.00E-91
PN138	MP1 [Cymbidium mosaic virus]	131	3.00E-30
PN139	elongation factor 1 alpha [*Pyrus pyrifolia*]	144	5.00E-34
PN140	triple gene block 2 [Cymbidium mosaic virus]	248	6.00E-65
PN141	RNA replicase [Cymbidium mosaic virus]	158	3.00E-38
PN142	triple gene block 1 [Cymbidium mosaic virus]	419	e-116
PN143	RNA dependent RNA polymerase [Cymbidium mosaic virus]	443	e-123
PN144	endomembrane protein 70, putative [*Arabidopsis thaliana*]	302	5.00E-81
PN145	triple gene block 2 [Cymbidium mosaic virus]	35.8	0.17
PN147	coat protein [Cymbidium mosaic virus]	109	8.00E-24
PN148	triple gene block 1 [Cymbidium mosaic virus]	351	e-102
PN149	triple gene block 2 [Cymbidium mosaic virus]	89.7	8.00E-18
PN150	S-adenosyl-L-methionine decarboxylase [*Dendrobium crumenatum*]	138	3.00E-32
PN151	RNA replicase [Cymbidium mosaic virus]	328	2.00E-88

[a]Sequence description of the highest scoring alignments described by one line summaries

[b]Scores (bits) assigned to an alignment calculated by summing the scores normalizing with the statistical variables for each position in the alignment.

[c]Expect (E) value describes the statistical significance threshold for reporting matches against database sequences.

Tab. 7 Annotation of cDNA clones enriched in peloric flower buds from SSH flower-leaves analysis

Clone ID	Description[a]	Score (bits)[b]	E value[c]
D1	Tubulin beta-2 chain [Beta-2 tubulin]	114	5.00E-25
D3	hypothetical protein XP_155099 [*Mus musculus*]	31.2	3.8
D4	prunasin hydrolase isoform PHA precursor [*Prunus serotina*]	218	7.00E-56
D5	water channel-like protein [*Arabidopsis thaliana*]	139	1.00E-32
D6	similar to Spindlin-like protein 2 (SPIN-2) [*Rattus norvegicus*]	36.2	0.35
D 7	unknown protein [*Oryza sativa*]	209	3.00E-53
D 8	unknown protein [*Oryza sativa*]	209	3.00E-53
D9	early light-induced protein-like protein [*Retama raetam*]	158	6.00E-38
D10	early light-induced protein-like protein [*Retama raetam*]	158	6.00E-38
D12	alpha tubulin [*Physcomitrella patens*]	233	1.00E-60
D13	putative RNase [*Oryza sativa* (japonica cultivar-group)]	125	3.00E-28
D14	unknown protein (sp|P72777) -related [*Arabidopsis thaliana*]	80.9	8.00E-15
D15	lipid transfer protein homolog [*Triticum aestivum*]	73.2	9.00E-13
D16	silverleaf whitefly-induced protein 3 [*Cucurbita pepo*]	204	9.00E-52
D18	protease inhibitor/seed storage/lipid transfer protein (LTP) family [*Arabidopsis thaliana*]	35	0.26
D19	ribosomal protein RL5 [*Cicer arietinum*]	102	1.00E-21
D20	transcription factor PCF6 [*Oryza sativa* (japonica cultivar-group)]; TCP family transcription factor	49.7	4.00E-05
D21	hypothetical protein F8F16.250 – [*Arabidopsis thaliana*]	30.8	4.2
D22	Ferrochelatase II, chloroplast precursor (Protoheme ferro-lyase)	301	6.00E-81
D24	putative RNase H [*Arabidopsis thaliana*]	115	1.00E-25
D26	hypothetical protein [*Encephalitozoon cuniculi*]	32	3.7
D27	unknown [*Zea mays*]	87.8	3.00E-17
D28	hypothetical protein [swollen duckweed]	69.3	1.00E-11
D29	cellulose synthase-7 [*Zea mays*]	310	9.00E-84
D31	pectinesterase family [*Arabidopsis thaliana*]	135	1.00E-31
D32	hypothetical protein [*Bacteroides thetaiotaomicron* VPI-5482]	31.2	3.8
D35	polyamine oxidase [*Hordeum vulgare* subsp. vulgare]	224	1.00E-57
D38	plastid-lipid associated protein PAP/fibrillin family [*Arabidopsis thaliana*]	147	1.00E-57
D39	CG8146-PA [*Drosophila melanogaster*]	31.6	3.2
D42	expressed protein [*Arabidopsis thaliana*]	137	9.00E-32
D43	GLP_78_3038_1476 [*Giardia lamblia* ATCC 50803]	32	2.4
D44	plasma membrane aquaporin [*Vitis vinifera*]	209	8.00E-54
D45	late embryogenesis abundant protein – [*Arabidopsis thaliana*]	74	4.00E-13
D46	AtHVA22a [*Arabidopsis thaliana*]	55.5	2.00E-07
D47	unknown protein [*Arabidopsis thaliana*]	146	3.00E-34
D48	GDSL-motif lipase/hydrolase protein [*Arabidopsis thaliana*]	177	1.00E-43
D49	histone H2A [*Euphorbia esula*]	167	5.00E-41
D50	GDSL-motif lipase/hydrolase protein [*Arabidopsis thaliana*]	168	5.00E-41
D52	AtHVA22a [*Arabidopsis thaliana*]	55	2.00E-07
D54	beta-tubulin [*Cicer arietinum*]	55	2.00E-07
D55	unknown protein [*Oryza sativa*]	194	1.00E-48
D56	phospholipid transfer protein [*Aerides japonica*]	166	7.00E-41
D57	arginine decarboxylase 2 [*Nicotiana tabacum*]	172	7.00E-43
D58	unknown protein [*Oryza sativa*]	142	7.00E-43
D60	GDSL-motif lipase/hydrolase protein [*Arabidopsis thaliana*]	177	8.00E-44
D61	putative carboxymethylenebutenolidase [*Oryza sativa*]	139	6.00E-33
D63	GDSL-motif lipase/hydrolase protein [*Arabidopsis thaliana*]	176	2.00E-43
D64	GDSL-motif lipase/hydrolase protein [*Arabidopsis thaliana*]	114	3.00E-25
D65	conserved hypothetical protein [*Borrelia burgdorferi* B31]	32	2.6
D66	proline-rich-like protein [*Asparagus officinalis*]	51.2	3.00E-06
D70	tocopherol cyclase [*Eucalyptus gunnii*]	163	5.00E-40
D71	acyltransferase homolog [*Petunia x hybrida*]	134	8.00E-31

Tab. 7 Annotation of cDNA clones enriched in peloric flower buds from SSH flower-leaves analysis (continued-1)

Clone ID	Description[a]	Score (bits)[b]	E value[c]
D78	P0485B12.26 [*Oryza sativa* (japonica cultivar-group)]	75.1	4.00E-13
D79	protein kinase family [*Arabidopsis thaliana*]	153	2.00E-36
D80	putative 60S ribosomal protein L44 [*Oryza sativa*]	201	2.00E-51
D81	chloroplast ribosomal protein S22 [*Spinacia oleracea*]	115	2.00E-25
D82	lipid transfer protein precursor [*Davidia involucrata*]	68.6	2.00E-11
D83	putative ribosomal protein L19 [*Oryza sativa*]	129	2.00E-29
D84	P0705A05.30 [*Oryza sativa*]	213	2.00E-62
D85	P0498E12.14 [*Oryza sativa*]	68.2	6.00E-11
D86	senescence-associated protein family [*Arabidopsis thaliana*]	96.7	2.00E-19
D89	lipid transfer protein precursor [*Davidia involucrata*]	105	1.00E-22
D90	phospholipid transfer protein [*Aerides japonica*]	202	1.00E-51
D91	lipid transfer protein precursor [*Davidia involucrata*]	105	1.00E-22

[a]Sequence description of the highest scoring alignments described by one line summaries

[b]Scores (bits) assigned to an alignment calculated by summing the scores normalizing with the statistical variables for each position in the alignment.

[c]Expect (E) value describes the statistical significance threshold for reporting matches against database sequences.

terference remains to be elucidated. Elimination of false positive clones during SSH has been reported by using a Mirror Orientation Selection [42].

Expression levels of selected ESTs in wild type and mutant flowers

In order to confirm the differential expression of the cloned ESTs from both cDNA-RAPD and SSH, real-time RT-PCR using SYBR Green as fluorescence source was conducted to analyze selected clones to check their relative expression level in either wild type or peloric or semi-peloric flower buds by comparing to the housekeeping gene. These clones were selected due to their probable role in cell division, hormone induction, and flower development. First, standard curves of the housekeeping gene, *actin*, and then selected target genes were generated with the ABI Prism 7900 detection system. Coefficients of determination (R^2) for the standard curves were more than 0. 96 for most ESTs examined, except for the clones ZOPB10M-60-2(1) and LOPW06N-11-3(19), but all above 0.82-0.91 (Fig. 4).

Expression level of the selected clones was shown in Fig. 5, except of one WD-40 repeat clone OPT05M-50 (Tab. 3) which did not show significant difference between wild type and mutants (data not shown). The clone LOPW02N-3-2(3), encoding a TGA1a-like protein belonged to the bZIP family, was 3-fold and 1.4-fold up-regulated in the wild type as compared to the peloric mutant and semi-peloric mutant respectively (Fig. 5A). The clone LOPW06N-11-3(19) was slightly up-regulated in the peloric and semi-peloric mutants than the wild type (Fig. 5B). The Clone LOPW07N-13(29) was down-regulated in the semi-peloric flower buds as compared to the wild type and the peloric (Fig. 5D). Two clones, ZOPAA01M-2(72) and 607FD12, were highly up-regulated in the prloric mutants. The ZOPAA01M-2(72) clone is a CyMV RNA-dependent RNA polymerase, which was expressed only at basal level in the wild type and semi-peloric flower buds, but highly in the peloric mutants (Fig. 5C). The other highly expressed clone 607FD12 is a retroelement, which was also 6-fold expressed in the peloric mutant than the wild type (Fig. 5E). An unknown protein clone LOPW02M-4-1(7) was down-regulated in the semi-peloric flower buds, and about 1.6-fold expressed in the peloric flower buds and 1.3-fold in the wild type (Fig. 5F). ZOPB10M-60-2 (1) obtained from cDNA-RAPD represents an auxin-regulated dual specificity cytosolic kinase. It was highly expressed in the peloric mutants, with 3-fold higher than the wild type and about 1.8 fold-in semi-peloric mutants (Fig. 5G). A serine/threonine protein kinase gene *APK2a* was reported to be negatively regulated by the AGAMOUS protein in flower development [43]. The Arabidopsis receptor-like kinase ERECTA also plays a role in inflorescence architecture [44]. Finally, clone ZOPAA12M-24(65) from cDNA-RAPD, encoding a cyclophilin-like protein, was slightly up-regulated in the peloric mutants (Fig. 5H). One clone isolated from SSH experiment with 1.6-fold increase in transcripts in the peloric mutants has sequence similarity to a transcriptional factor PCF6 and a TCP family member (Fig. 5I).

DISCUSSION

SSH has been reported as a tool to compare differentially expressed genes during development and in responses to stresses, pests and somaclonal variations [31, 45]. Since many cultivated orchids tended to be infected by different

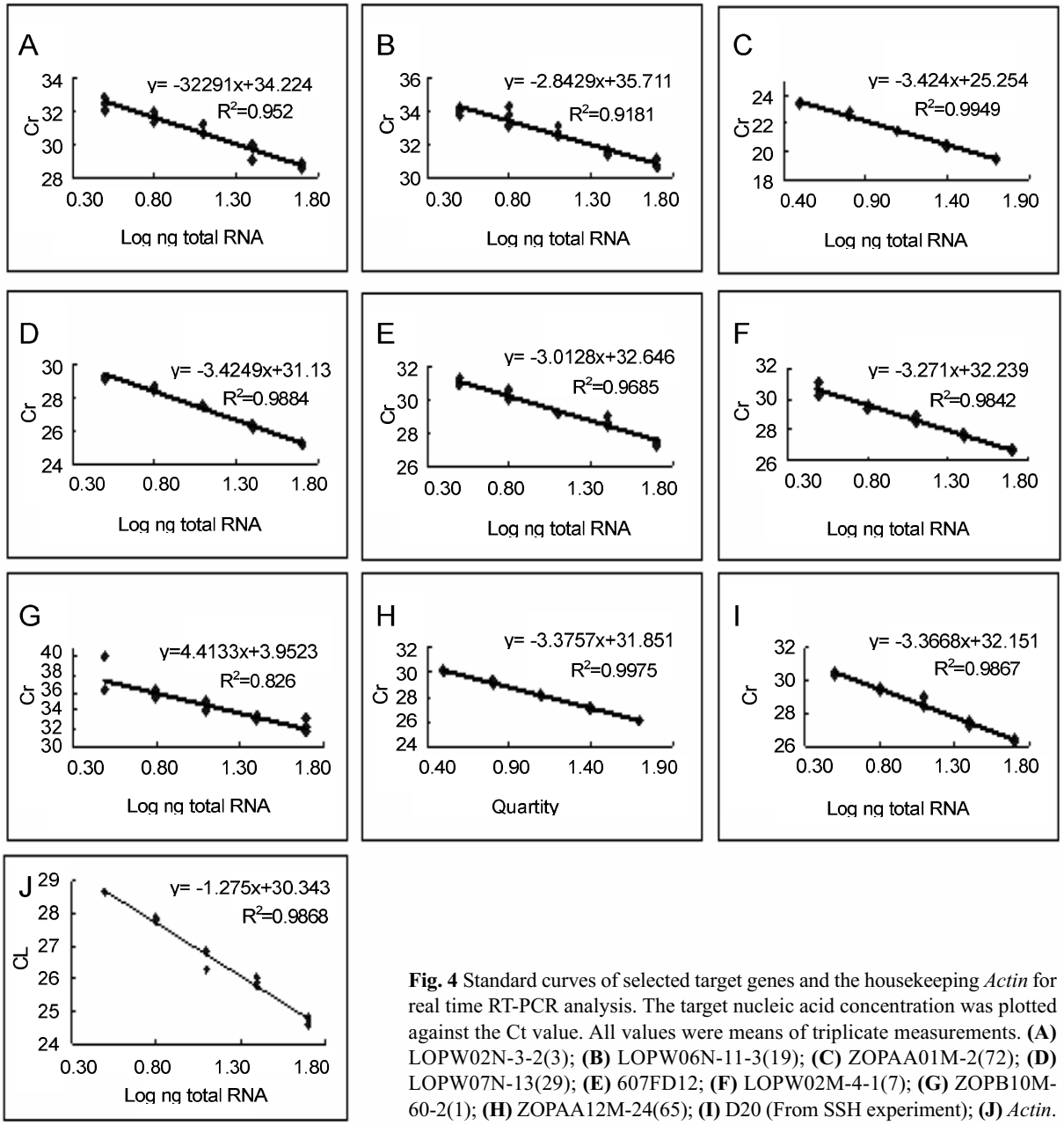

Fig. 4 Standard curves of selected target genes and the housekeeping *Actin* for real time RT-PCR analysis. The target nucleic acid concentration was plotted against the Ct value. All values were means of triplicate measurements. **(A)** LOPW02N-3-2(3); **(B)** LOPW06N-11-3(19); **(C)** ZOPAA01M-2(72); **(D)** LOPW07N-13(29); **(E)** 607FD12; **(F)** LOPW02M-4-1(7); **(G)** ZOPB10M-60-2(1); **(H)** ZOPAA12M-24(65); **(I)** D20 (From SSH experiment); **(J)** *Actin*.

viruses, such as the CyMV and ORSV [46]), the abundant virus transcripts in the plant cells and tissues might interfere with the SSH or the cDNA-AFLP analysis [47]. In this study, although limited numbers of differentially expressed ESTs were obtained, several interesting clones derived from both suppression subtractive hybridization and cDNA-RAPD analyses revealed potential roles in flower development. A big portion of the subtracted clones belonged to the orchid virus, although it is not clear if the virus infection may cause the phenotypic change. There are clones differentially expressed in the mutant flower buds and one of the most interesting ones is D20, which encodes a potential TCP domain protein. The study on bilateral symmetry in snapdragon flower showed that the radial symmetry was caused by mutations in *cycloidea* and *dichotoma* genes [48, 49]. Both gene products belong to the members of the TCP family proteins that control plant development from different aspects [39-41]. We have attempted to clone the *cycloidea* (*cyc*) homolog in *Phalaenopsis* orchids by using degenerate primers based on conserved amino acid sequence of *cyc* homologs, but failed (data not shown). The cloning of TCP homolog, D20 from peloric flower buds by SSH analysis (Tab. 7) enables us to further characterize the biological function

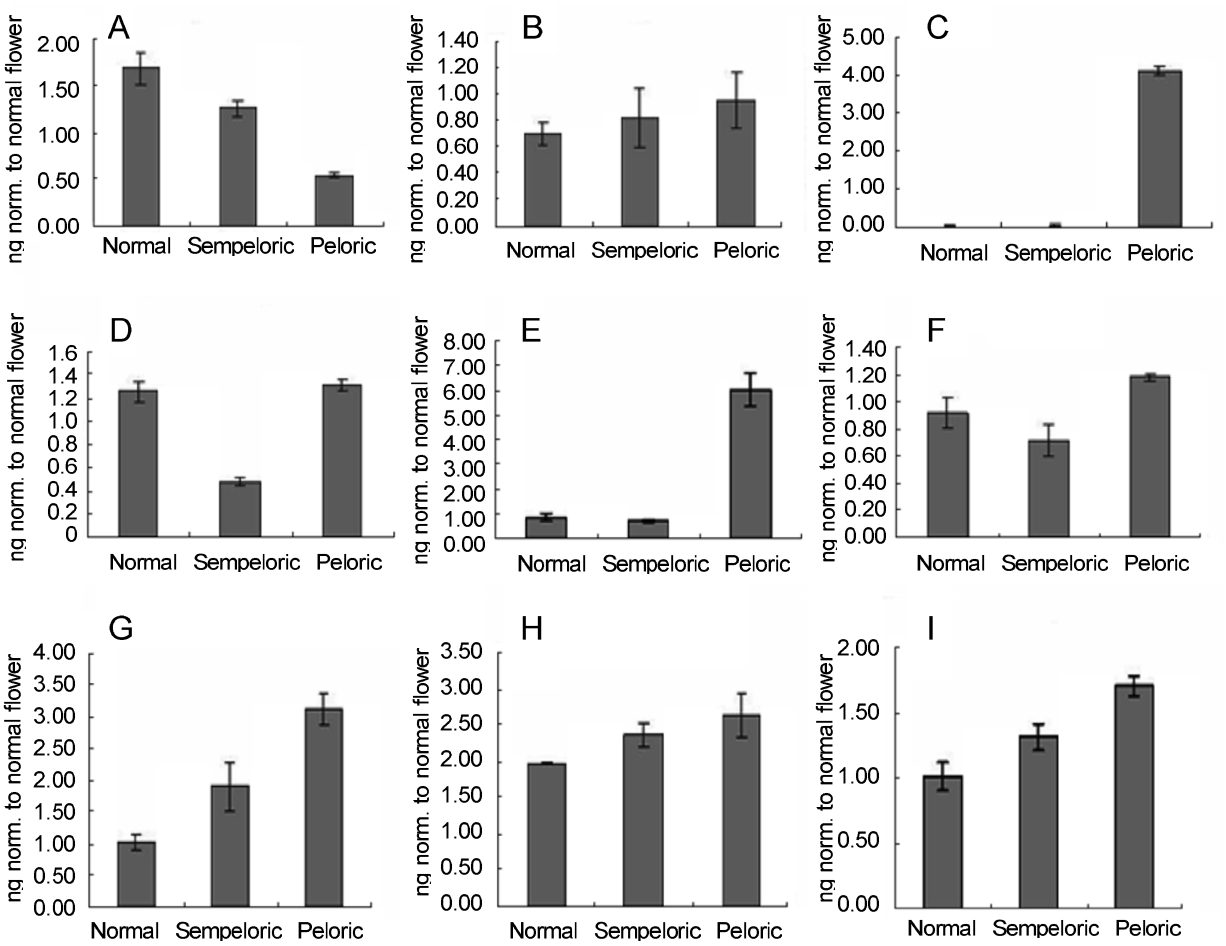

Fig. 5 Real-time RT-PCR analysis for expression levels of selected target genes of normal (wild type) and mutant flower buds of *Phalaenopsis* orchids from both cDNA-RAPD and suppression subtractive hybridization. **(A)** LOPW02N-3-2(3), encoding a TGA1a-like protein; **(B)** LOPW06N-11-3(19), encoding a protein kinase; **(C)** ZOPAA01M-2(72), encoding CyMV RNA-dependent RNA polymerase; **(D)** LOPW07N-13(29), encoding a praline iminopeptidase; **(E)** 607FD12, a retroelement; **(F)** LOPW02M-4-1(7), encoding an unknown protein; **(G)** ZOPB10M-6-2(1), encoding auxin-regulated dual specificity cytosolic kinase; **(H)** ZOPAA12M-24(65), encoding cyclophilin-like protein; **(I)** D20, encoding a transcriptional factor PCF6 or TCP family protein.

of D20 in floral symmetry in *Phalaenopsis* orchids.

Bulge of the central part of lateral petals in the peloric flowers might be epidermal cell origin. The interaction between transcription factor such as the homeobox genes and TCP homolog and some other protein factors may lead to ectopic cell division in the petals to form the bulge structure [45, 50]. One homoebox gene was recently cloned from the phalaenopsis inflorescence by using degenerate primers based on multiple nucleotide sequence alignment. The homeobox shows homology to the ovule-specific homeobox gene [51] and a rice *GL2*-type homeobox gene *Roc1* specifically expressed in the protoderm or epidermal layer of rice embryo [52]. Stimuli of hormones, such as the auxin-regulated dual specificity cytosolic kinase may induce unusual cell division in the bulge regions of the peloric petals as shown in Fig. 5G. The unequal distribution of auxin may lead to unusual cell division in certain part of a leaf tissue [53], which may imply similar situation in phalaenopsis flowers. The leaf of the asymmetric leaves 1 mutant of Arabidopsis was reverted by applying auxin onto the leaves, an indication of the role of polar auxin transport in leaf patterning [53]. The putative auxin-regulated gene also shares high homology to serine/threonine/tyrosine-specific protein kinases, suggesting a role in the signal transduction pathway during cell division or plant development [54-56].

In conclusion, we have obtained hundreds of ESTs from the wild type and peloric mutant flower buds of the Phalaenopsis hybrids by using techniques of cDNA-RAPD and SSH. Biased as well as redundant clones towards the wild type or the peloric mutants revealed potential differential transcription regulation. Several analysed ESTs, such

as the retroelement, TCP family proteins, auxin-regulated kinase may play a role in the orchid flower development. One question we can ask is whether these up-regulated ESTs due to the epigenetic regulation mechanism, such as DNA hypomethylation? DNA methylation has been implied as a mechanism regulating plant development such as vernalization and somaclonal variation [18, 23, 24, 57-60]. Recently, we have cloned the full length cDNA of a DNA cytosine methyltransferase and a receptor-related protein kinase, ERECTA-like gene from the SSH study (Tab. 5). Their function in orchid flower development will be further characterized through complementation of Arabidopsis mutants and via knockout orchids by genetic approach.

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