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Identification of warm day and cool night conditions induced flowering-related genes in a *Phalaenopsis* orchid hybrid by suppression subtractive hybridization

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ABSTRACT. The influence of warm day and cool night conditions on induction of spikes in *Phalaenopsis* orchids has been studied with respect to photosynthetic efficiency, metabolic cycles and physiology. However, molecular events involved in spike emergence induced by warm day and cool night conditions are not clearly understood. We examined gene expression induced by warm day and cool night conditions in the *Phalaenopsis* hybrid Fortune Saltzman through suppression subtractive hybridization, which allowed identification of flowering-related genes in warm day and cool night conditions in spikes and leaves at vegetative phase grown under warm daily temperatures. In total, 450 presumably regulated expressed sequence tags (ESTs) were identified and classified into functional categories, including metabolism, development, transcription factor, signal transduction, transportation, cell defense, and stress. Furthermore, database comparisons revealed a notable number of *Phalaenopsis* hybrid Fortune Saltzman ESTs that matched genes with

unknown function. The expression profiles of 24 genes (from different functional categories) have been confirmed by quantitative real-time PCR in induced spikes and juvenile apical leaves. The results of the real-time PCR showed that, compared to the vegetative apical leaves, the transcripts of genes encoding flowering locus T, AP1, AP2, KNOX1, knotted1-like homeobox protein, R2R3-like MYB, adenosine kinase 2, S-adenosylmethionine synthetase, dihydroflavonol 4-reductase, and naringenin 3-dioxygenase accumulated significantly higher levels, and genes encoding FCA, retrotransposon protein Ty3 and C3HC4-type RING finger protein accumulated remarkably lower levels in spikes of early developmental stages. These results suggested that the genes of two expression changing trends may play positive and negative roles in the early floral transition of *Phalaenopsis* orchids. In conclusion, spikes induced by warm day and cool night conditions were complex in *Phalaenopsis* orchids; nevertheless, several molecular flowering pathway-related genes were found. The acquired data form the basis for a molecular understanding of spike induction by warm day and cool night conditions in *Phalaenopsis* orchids.

Key words: *Phalaenopsis*; Flowering gene; Spike induction; Gene expression; Flowering locus T

INTRODUCTION

Transition from vegetative to reproductive growth is one of the most important developmental changes in the angiosperm life cycle. The transition is controlled by both internal signals and external environmental factors, which later integrate to result in flowering. In the dicot model *Arabidopsis*, for example, five genetically defined pathways - photoperiod, vernalization, gibberellin, autonomous, and aging - have been identified in the control of flowering (Srikanth and Schmid, 2011). The photoperiod pathway refers to regulation of flowering in response to day length and quality of light perceived, whereas the vernalization pathway monitors flowering on exposure to a long period of low temperature. The gibberellin pathway promotes flowering requirement of gibberellic acid for normal flowering patterns. The autonomous pathway refers to endogenous regulators that are independent of the photoperiod and gibberellin pathways, and the aging pathway, where plant age controls flowering time (Srikanth and Schmid, 2011). The flowering signals from these multiple genetic pathways ultimately converge on the regulation of two major floral pathway integrators, *FT* (*flowering locus T*) and *SOC1* (*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*), which in turn activate floral meristem identity genes, such as *AP1* (*APETALA1*) and *LFY* (*LEAFY*), to initiate the generation of floral meristems (Abe et al., 2005; Corbesier et al., 2007; Lee et al., 2008; Lee and Lee, 2010).

Phalaenopsis, or moth orchid, belongs to Orchidaceae, one of the largest angiosperm families. It is a very popular and the most valuable potted plant in the world because of its stylish, elegant appearance and extended flowering period. Species of this genus are epiphytic crassulacean acid metabolism (CAM) plants (Endo and Ikusima, 1989; Guo and Lee, 2006), originating from tropical and subtropical areas of the South Pacific Islands and Asia. Their juvenile phase lasts up to 36 months from seeding for many commercial *Phalaenopsis* hybrids (Hew and Yong,

1997), which makes it difficult to meet specific market dates. Several studies on the regulation of flowering in *Phalaenopsis* orchids are mainly concern the effects of temperature and photoperiod (Chen et al., 1994, 2008; Sakanishi et al., 1980; Wang, 1995). Moreover, compared with photoperiod, temperature is a more important environmental factor for inducing spikes in *Phalaenopsis* orchids. For commercial production, *Phalaenopsis* hybrids are always treated with low temperatures in temperature-controlled greenhouses or cultivated in naturally cool environments such as in high mountains to accelerate floral transition. During greenhouse production, the recommended average daily temperatures of 25°-30°C are maintained to promote leaf development and inhibit flower initiation (Chen et al., 1994; Sakanishi et al., 1980). Conversely, fluctuating warm day (28°C) and cool night (20°C) conditions significantly induces the formation of spikes of *Phalaenopsis* plants (Chen et al., 2008). Furthermore, the effects of warm day and cool night conditions on *Phalaenopsis* plants have been studied with respect to photosynthetic efficiency, metabolic pools and physiology. For example, on the basis of the characteristics of CAM plants, warm day and cool night conditions affect photosynthetic efficiency, metabolizable carbohydrate and organic acid pools of *Phalaenopsis* orchids (Chen et al., 2008; Pollet et al., 2011a,b).

Although a number of expressed sequence tags (ESTs), transcriptomic and smRNA sequences obtained from *Phalaenopsis* reproductive organs have been deposited in GenBank or OrchidBase (Chen et al., 2005; Tsai et al., 2006; An et al., 2011; Hsiao et al., 2006, 2011), only a few of these genes and smRNAs have been characterized. Examples are *ORAP11*, *ORAP13*, *CONSTANS*-like, and *FVE* from *Phalaenopsis* orchids (*Doritaenopsis* included), which are believed to play important roles in floral transition and regulation of flowering time (Chen et al., 2007; Zhang et al., 2011; Sun et al., 2012). However, the molecular mechanisms of warm day and cool night conditions for flowering initiation in *Phalaenopsis* orchids, especially identification of central genes regulating flowering time as well as those controlling other processes affecting flowering, have not been well understood so far.

In the present study, we principally focused our attention on the identification of flowering-related genes in spikes of *Phalaenopsis* orchids induced by warm day and cool night conditions. One suppressive subtractive hybridization (SSH) cDNA library was constructed to compare genes differentially expressed between spikes induced by warm day and cool night conditions and juvenile apical leaves grown under warm daily temperatures. We searched *Phalaenopsis* homologs with known flowering genes by EST and bioinformatic analysis. A group of putative flowering genes corresponding to known genetic pathways regulating flowering time were identified. Among these genes, several genes were chosen for expression analysis of their spatial and temporal patterns between induced spikes and juvenile apical leaves. Information gathered on these genes is discussed and suggests that they can play important roles in floral transition induced by fluctuating warm days and cool nights.

MATERIAL AND METHODS

Plant material and growth conditions

In August 2010, mature, non-flowering plants with at least 5 fully developed leaves of *Phalaenopsis* hybrid Fortune Saltzman were purchased from a commercial farm (Flower Star, Foshan, Guangzhou, China), and grown in 2.5-inch diameter plastic pots filled with sphagnum moss in a greenhouse at the Environmental Horticulture Research Institute, Guangdong Academy

of Agricultural Sciences, under natural light and controlled temperature. Prior to the experiment, plants were placed in a greenhouse for at least 2 weeks with day (70-95% relative humidity, RH)/night (100% RH) temperature regime of 28° ± 2°C. Plants comprising the untreated group were subsequently grown at day (28° ± 2°C)/night (26° ± 1.5°C) (day 70-95% RH/night 100% RH) to inhibit phase transition. Warm day and cool night treatment was carried out at day (28° ± 2°C)/night (21° ± 1.5°C) (day 70-95% RH/night 100% RH) for 1.5 months to complete phase transition.

Position and timing of sampling

The apical leaves without cool night treatment were collected at 2-week intervals from 0 to 1.5 months. Reproductive stems, *viz*. spikes, were only present in the warm day and cool night treatment group. For *Phalaenopsis* hybrid Fortune Saltzman, spikes were collected at 0-3, 3-5, 5-10, 10-15, 15-20, 20-35, and 40 mm in length, separately. Each sample was pooled from 3 separate plants and stored separately for all the experiments in this study.

RNA extraction

Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen, Germany) according to manufacturer protocols. Total RNA was then treated by RNase-free DNase I (TaKaRa, Japan) to remove DNA contamination. The total RNA yield and quality were determined spectrophotometrically at wavelengths of 230, 260 and 280 nm. The integrity of total RNA was checked by running samples on 1.2% denaturing agarose gels.

Amplification by SMART technology and construction of subtracted complementary DNA (cDNA) library

For SSH library, total RNA was extracted from spikes at 40 mm in length from the warm day- and cool night-treated group and vegetative apical leaves pooled at different untreated times of *Phalaenopsis* hybrid Fortune Saltzman. Total RNA (1.0 μg per sample) was reverse-transcribed and amplified to produce SMART cDNA using the SMART™ PCR cDNA Synthesis kit (Clontech, USA) according to the user manual.

The subtractive cDNA library was obtained using the PCR-select cDNA subtraction kit (Clontech). The tester (warm day- and cool night-induced spikes) SMART-cDNA sample was subtracted twice by the driver (untreated apical leaves) SMART-cDNA sample following manufacturer recommendations (forward subtraction). Subtracted cDNA sequences were then immediately ligated into the pGEM-T easy vector (Promega, USA), and transformed into competent cells of *Escherichia coli* DH5α (TIANGEN, China). Transformed cells were seeded onto LB agar plates containing 100 μg/mL ampicillin, 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and 80 μg/mL X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), then incubated at 37°C overnight for blue-white colony screening. Individual recombinant white colonies were picked and grown in LB liquid medium containing 50 μg/mL ampicillin.

cDNA sequencing and sequence data analysis

Plasmids containing cDNA fragments were prepared on the Applied Biosystems

3730 DNA analyzer (Shanghai, Invitrogen, China). The sequences were trimmed to eliminate vector and adaptor sequences prior to further analysis. Homology searches of all sequences were queried in the GenBank database by using the BLASTn and BLASTx search at the NCBI network service (http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al., 1997). The ESTs with significant matches using cut-off value 1e-5 were classified into functional categories according to the Gene Ontology database (http://www.geneontology.org). Sequence data of *Phalaenopsis* hybrid Fortune Saltzman have been submitted to the GenBank under the accession numbers indicated below.

Analysis of differential ESTs by real-time PCR

Differential EST expression levels in the spatial and temporal distribution of the juvenile apical leaves and spikes (0-3, 3-5, 5-10, 10-15, 15-20, and 20-35 mm) were detected by real-time PCR. cDNA of each sample was synthesized using the PrimeScript™ First-Strand cDNA Synthesis kit (TaKaRa) according to the manufacturer protocol. Primers for differential ESTs were designed with Prime Primer 5 (Table 1). Real-time PCR was carried out with SYBR Green I kit (TaKaRa) in a final volume of 20 μL, including 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 10 μL SYBR Green Premix (2X), 2.0 μL diluted first-strand cDNA and 7.0 μL sterile distilled water. The reactions were preformed in the Light Cycler 480 real-time PCR system (Roche Diagnostics, USA) using the following program: preheating at 95°C for 30 s followed by 40 cycles of 5 s at 95°C, 15 s at 58°C and 30 s at 72°C. The levels of gene expression were analyzed with the Light Cycler 480 Software (Roche Diagnostics) and normalized with the results for *actin* (AY134752). Real-time PCR was performed in three replicates for each sample, and data are reported as means ± SD (N = 3).

Table 1. Primer sequences used for quantitative real-time PCR.

Clone No.	Forward primer (5'→3')	Reverse primer (5'→3')	Product length (bp)
FS33	TGTGAAGGAGCGGCTTGATT	CTTCCTTCGCCTTCTTGATA	215
FS71	TGCTGAGTATGAGGGCTTGA	TCGCTTCGGACTTTCCAACT	129
FS82	TTTGCTTGACATTCCACCAG	TTTCCATACCCTCCTTTCTT	176
FS89	CTATGCCCTCAGTTCTACCG	AGGTGGCAGTCCATCAGTCG	248
FS184	CTTCATTTAGGCGGTTCCAG	AGTGGAAGAACCTGGCTCAA	138
FS210	CCCACCCTATTGACCGAGTT	GTGCCCGAACTCATCCAAGC	147
FS276	GCAATCCTCCGCTCGCCTAC	CTCTTATGCCCGACCTCTTG	153
FS278	CAATCCAAAGACCCTGAGA	CTCAGGATGCCAAGCAAACC	65
FS327	TCAAGTCGGCGGTCTCCAAG	AATCGACCGTTGCTCAGGTA	123
FS344	AACGAGGGACACCCAGACAA	GAAACCGATGGAACGACAGG	201
FS383	GACAACATCTGCGGTCTGGC	TTTGAACCTCCACAGCACTT	147
FS397	GATGTGCCATTGGTCAGAAG	AACACTTCGCCAAACCTAAA	193
FS449	TCCTCAGTCATTTGGGTTCT	AGCAAATAATCGCTCACAGG	286
FS469	CAATCTGTCTAATCGCTCAC	GTAATGCCACGCCCTCCCAC	101
FS494	CTATTGGGCACCTTTCCTTG	CACAGAGCCTTTCATCCTAC	180
FS527	ACCGCTTTGTCTTCGTGCTG	CGACTGGCGAACCGAGATTA	120
FS652	ACAAGTTTATCTGGGAGGTT	ATTCTCCAGAGGGAAGTTGA	112
FS715	TTTCATAGGTCCGCAAGCAG	GTTGGTGCTGGTTCGGTTGG	111
FS789	CAGGGAGAACTGACAACGAA	GGATGGCAGTGCTTGATTTG	113
FS814	TGAAGGCAAAGGTTGAAAGC	TGCTGGTAGGAGTTGGGACA	342
FS834	CTCGGTTGGCATTGGTCATA	CATTTACACCTGCTTCTTTA	351
FS681	CGAGCCTGAAAGTGTTAGAT	TGATGAAAGTCCAGCAAGAT	163
FS899	GGGACTTTCTATCGGCTTAC	CTCACTTCCACTGCCACCAC	181
FS928	CCGCAGAAGAAGAAATGAAA	CGAGCAGCGATACTATGTCC	117
Actin	CAGTGTTTGGATTGGAGGTT	TCTCGGGTTCCATTTCCATC	139

RESULTS

Construction of the subtracted cDNA library

To identify the warm day- and cool night-induced flowering genes, an SSH library, which was enriched for cDNAs corresponding to warm day- and cool night-induced flowering genes, was constructed. The cDNA fragments obtained were subcloned into a pGEM-T easy vector. A total of 650 white colonies were randomly isolated from LB-ampicillin medium. Each colony was used to amplify the inserted sequences. Most of the inserts ranged from 200 to 1200 bp. After editing, 450 non-redundant ESTs exhibited significant homology to plant genes. On the basis of their putative functions, the ESTs were annotated and classified into eleven categories as follows: metabolism, development, cell defense and stress, transcription regulation, signal transduction, transportation, protein synthesis and modification, photosynthesis, cell structure and component, energy, unknown and no hit proteins (Table 2). The metabolism category (consisting of six sub-classes: amino acid, C-compound, nucleotide, lipid and fatty acid, phosphate, and secondary metabolism) contained 98 distinct deduced proteins from 107 ESTs, which accounted for 23.78% (107/450) of the classified ESTs. The category protein synthesis and modification contained 53 distinct proteins deduced from 53 ESTs, which accounted for 11.78% of the classified ESTs. Other categories were as follows: development (15 distinct proteins; 15 ESTs; 3.33%), cell defense and stress (23 distinct proteins; 24 ESTs; 5.33%), transcription regulation (24 distinct proteins; 24 ESTs; 5.33%), signal transduction (48 distinct proteins; 48 ESTs; 10.67%), transportation (17 distinct proteins; 18 ESTs; 4.00%), photosynthesis (9 distinct proteins; 9 ESTs; 2.00%), cell structure and component (31 distinct proteins; 33 ESTs; 7.33%), energy (16 distinct proteins; 19 ESTs; 4.22%), unknown and no hit protein (100 ESTs; 22.22%). Putative functions of all ESTs were annotated according to the highest scoring matches by BLAST in NCBI. Four hundred and fifty ESTs were deposited in NCBI dbEST database of GenBank with accession numbers of JK720314-JK720763. Some of the ESTs deposited in GenBank under the accession numbers are shown in Table 3.

Table 2. Functional categories of flowering-related ESTs induced by warm day and cool night conditions in *Phalaenopsis* hybrid Fortune Saltzman.

Functional classification	Number of ESTs	Ratio (%)
Metabolism	107	23.78
Amino acid metabolism	19	4.22
C-compound metabolism	18	4.00
Nucleotide metabolism	26	5.78
Lipid and fatty acid metabolism	9	2.00
Phosphate metabolism	4	0.89
Secondary metabolism	31	6.89
Development	15	3.33
Cell defence and stress	24	5.33
Transcription regulation	24	5.33
Signal transduction	48	10.67
Transportation	18	4.00
Protein synthesis and modification	53	11.78
Photosynthesis	9	2.00
Cell structure and component	33	7.33
Energy	19	4.22
Unknown and no hit protein	100	22.22
Total	450	100.00

Table 3. Genes differentially expressed in *Phalaenopsis* hybrid Fortune Saltzman spikes induced by warm day and cool night condition.

Clone No.	GenBank accession No.	Length (bp)	BLAST annotation (GenBank accession No.)	E value
Amino acid metabolism				
FS94	JK720363	149	Cysteine synthase [*Citrullus lanatus* subsp *vulgaris*] (BAA05965.1)	8e-21
FS344*	JK720495	520	S-adenosylmethionine synthetase [*Gossypium hirsutum*] (ADN96174.1)	2e-71
C-compound metabolism				
FS82*	JK720358	781	NAD-dependent malate dehydrogenase [*Prunus armeniaca*] (ADN87327.1)	1e-87
Nucleotide metabolism				
FS276*	JK720451	674	Caffeoyl-CoA 3-O methyltransferase [*Coffea canephora*] (ABO77959.1)	3e-86
FS465	JK720546	563	ATP-dependent RNA helicase, putative [*Ricinus communis*] (XP_002515245.1)	3e-51
Fatty acid and lipid metabolism				
FS944	JK720752	274	Long-chain-fatty-acid-CoA ligase family protein [*Arabidopsis lyrata* subsp *lyrata*] (XP_002880290.1)	2e-27
Phosphate metabolism				
FS588	JK720595	818	Putative purple acid phosphatase [*Arabidopsis thaliana*] (AAW29952.1)	1e-63
Secondary metabolism				
FS278*	JK720453	512	Dihydroflavonol 4-reductase [*Bromheadia finlaysoniana*] (AAB62873.1)	4e-80
FS327*	JK720481	1127	Naringenin 3-dioxygenase [*Bromheadia finlaysoniana*] (CAA61486.1)	0.0
FS383*	JK720510	393	Cytochrome P450 [*Zea mays*] (ACG38359.1)	1e-38
FS397*	JK720515	666	Sesquiterpene synthase [*Vanda* hybrid cultivar] (ABX57720.1)	4e-47
Signal transduction				
FS33*	JK720330	609	Early flowering protein 1 [*Elaeis guineensis*] (ACF06553.1)	3e-37
FS68	JK720352	529	Serine-threonine protein kinase, plant-type, putative [*Ricinus communis*] (XP_002530200.1)	2e-40
FS612	JK720609	704	Serine/threonine-protein kinase Nek4 [*Zea mays*] (NP_001152012.1)	6e-96
FS834*	JK720703	395	Adenosine kinase 2 [*Oryza sativa* Indica Group] (ABR25519.1)	2e-53
Transportation				
FS46	JK720339	385	PIP1_5/PIP1D [*Arabidopsis lyrata* subsp *lyrata*] (XP_002869782.1)	2e-45
FS311	JK720470	499	Potassium efflux antiporter [*Populus trichocarpa*] (XP_002308566.1)	2e-13
Energy				
FS716	JK720661	196	Vacuolar H^+-ATPase subunit C [*Gossypium hirsutum*] (ABH07428.1)	2e-23
FS752	JK720675	701	Putative DNA-dependent ATPase SNF2H [*Oryza sativa* Japonica Group] (BAD61441.1)	2e-51
Development				
FS184*	JK720404	451	KNOX1 [*Agave tequilana*] (ADN43388.1)	2e-27
FS527*	JK720571	809	Flowering locus T [*Cymbidium goeringii*] (ADI58462.1)	3e-90
FS652*	JK720623	583	AP2 domain-containing transcription factor [*Populus trichocarpa*] (XP_002302387.1)	2e-42
FS715*	JK720660	879	Flowering time control protein isoform OsFCA-2 [*Oryza sativa* Indica Group] (AAQ74972.1)	4e-31
FS814*	JK720695	603	AP1-related protein [*Phalaenopsis amabilis*] (AAZ76263.1)	3e-82
FS928*	JK720743	329	Knotted1-like homeobox protein [*Dendrobium* grex Madame Thong-In] (CAB88029.1)	6e-34
Transcription regulation				
FS71*	JK720353	466	Retrotransposon protein Ty3-gypsy subclass [*Zea mays*] (ACG35788.1)	1e-29
FS89*	JK720361	677	Helix-loop-helix DNA-binding domain protein [*Oryza sativa* Japonica Group] (NP_001051465.1)	3e-19
FS210*	JK720416	834	Zinc finger (C_3HC_4-type RING finger) family protein [*Arabidopsis thaliana*] (NP_564263.1)	1e-25
FS413	JK720519	521	MYB134 [*Glycine max*] (ABH02878.1)	5e-07
FS449*	JK720535	629	Putative NAC transcription factor [*Jatropha curcas*] (ADF59041.1)	1e-50
FS681*	JK720638	597	Transposon protein [*Zea mays*] (NP_001148044.1)	5e-88
FS789*	JK720689	672	MYB transcription factor R2R3-like protein [*Populus tremuloides*] (ACR83705.1)	4e-52
FS899*	JK720729	831	ARF domain class transcription factor [*Malus* x *domestica*] (ADL36581.1)	1e-46
Protein synthesis and modification				
FS376	JK720507	318	Asparaginyl endopeptidase [*Vigna radiata*] (AAK15049.1)	8e-39
FS469*	JK720548	242	Leucine-rich repeat-containing protein 40 [*Zea mays*] (ACG39716.1)	4e-09
Photosynthesis				
FS225	JK720425	338	Photosystem 1 subunit A [*Schisandra* sp SM-2010] (ADN44177.1)	2e-54

Continued on next page

Table 3. Continued.

Clone No.	GenBank accession No.	Length (bp)	Blast annotation (GenBank accession No.)	E value
Cell defence and stress				
FS14	JK720322	587	Mannose-specific lectin [*Dioscorea polystachya*] (BAD67184.1)	8e-19
FS40	JK720335	591	Class III peroxidase [*Oncidium* Gower Ramsey] (ABC02343.1)	3e-45
Cell component and structure				
FS49	JK720341	402	Cellulose synthase-like protein C10 [*Oryza sativa* Indica Group] (Q84Z01.1)	9e-65
Unkown and no hits protein				
FS32	JK720329	584	No hits	
FS379	JK720509	414	Unknown [*Zea mays*] (ACR36679.1)	2e-17
FS494*	JK720562	899	Unnamed protein product [*Vitis vinifera*] (CBI22809.3)	2e-67

*Clones selected for further real-time PCR analysis.

Gene expression by real-time PCR

To further confirm the expression profile of the genes obtained from SSH library, 24 genes belonging to various functional categories related to flowering transition, were examined in juvenile apical leaves and spikes induced by warm day and cool night conditions at different developmental stages using real-time PCR. The primer pairs of 24 genes are shown in Table 1. Compared with the levels in apical leaves, 21 genes showed upregulated levels in spikes at certain developmental stages, while expressions of 3 other genes slightly or remarkably decreased in spikes examined (Figure 1). These results indicated that the real-time PCR data agreed with the SSH data at 87.5%. Considering the inevitable technical errors introduced either from SSH construction or real-time PCR, it was concluded that overall there was a good agreement between the SSH data and real-time PCR results.

Real-time PCR also showed that 5 flowering-related ESTs (FS184, *KNOX1*; FS527, *FT*; FS814, *AP1*; FS652, *AP2*; FS928, *knotted1-like homeobox protein*) were significantly upregulated in induced spikes at 0-3 mm sizes (Figure 1). After spike development, expression profiles of these 5 genes showed diverse trends (Figure 1). The transcript levels of both *FT* and *AP1* were gradually reduced in tested spikes, and the transcripts of *KNOX1* were first consistent in spikes at 0-15-mm sizes, and then gradually decreased in spikes at 15-35-mm sizes, but the transcripts of these 3 genes were still higher in the spikes tested than in apical leaves. The expression patterns of *AP2* and *knotted1-like homeobox protein* showed fluctuating changes in the spikes tested. The transcripts of *AP2* were gradually decreased in 20-35-mm spikes and lower than in apical leaves, while the transcripts of *knotted1-like homeobox protein* were higher in spikes tested than in apical leaves. Interestingly, the transcript levels of one EST related to flowering time control protein FCA (FS715) in apical leaves were higher than in all the spikes tested, except in 15-20-mm spikes (Figure 1).

Other warm day- and cool night-induced genes confirmed by real-time PCR were involved in signal transduction (FS33, *early flowering protein 1*; FS834, *adenosine kinase 2*), metabolism (FS82, *NAD-dependent malate dehydrogenase*; FS276, *caffeoyl-CoA 3-O methyltransferase*; FS278, *dihydroflavonol 4-reductase*; FS327, *naringenin 3-dioxygenase*; FS344, *S-adenosylmethionine synthetase*; FS383, *cytochrome P450*; FS397, *sesquiterpene synthase*), transcription regulation (FS89, *Helix-loop-helix DNA-binding domain containing protein*; FS449, *NAC*; FS681, *transposon protein*; FS789, *R2R3-like MYB*; and FS899, *ARF domain class transcription factor*) and protein synthesis and modification (FS469, *leucine-rich repeat-containing protein 40*).

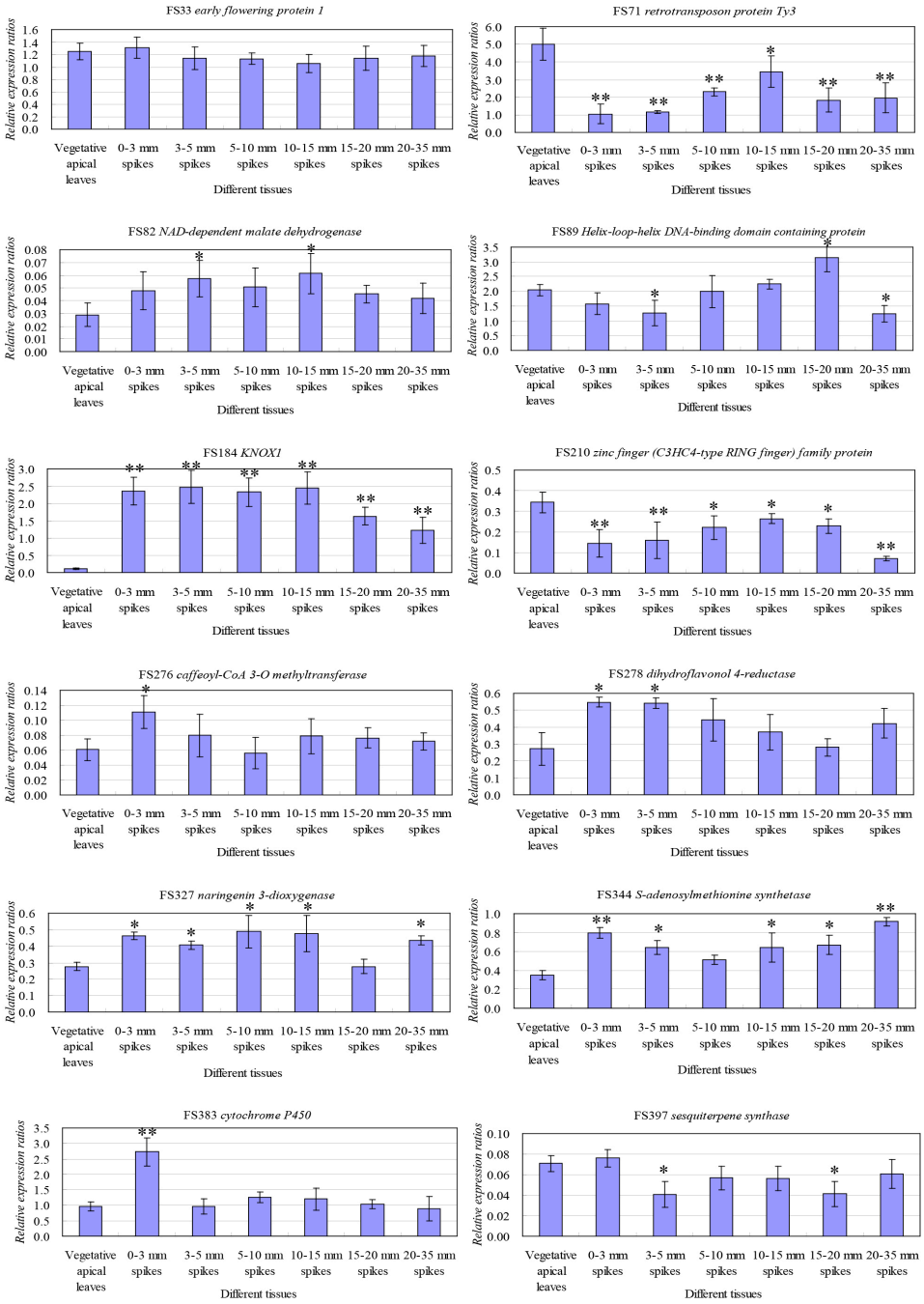

Figure 1. Results of real-time PCR for 24 genes obtained by suppression subtractive hybridization. All expression values were normalized to the values of the *actin* gene. Significance was indicated as *P < 0.05; **P < 0.01.

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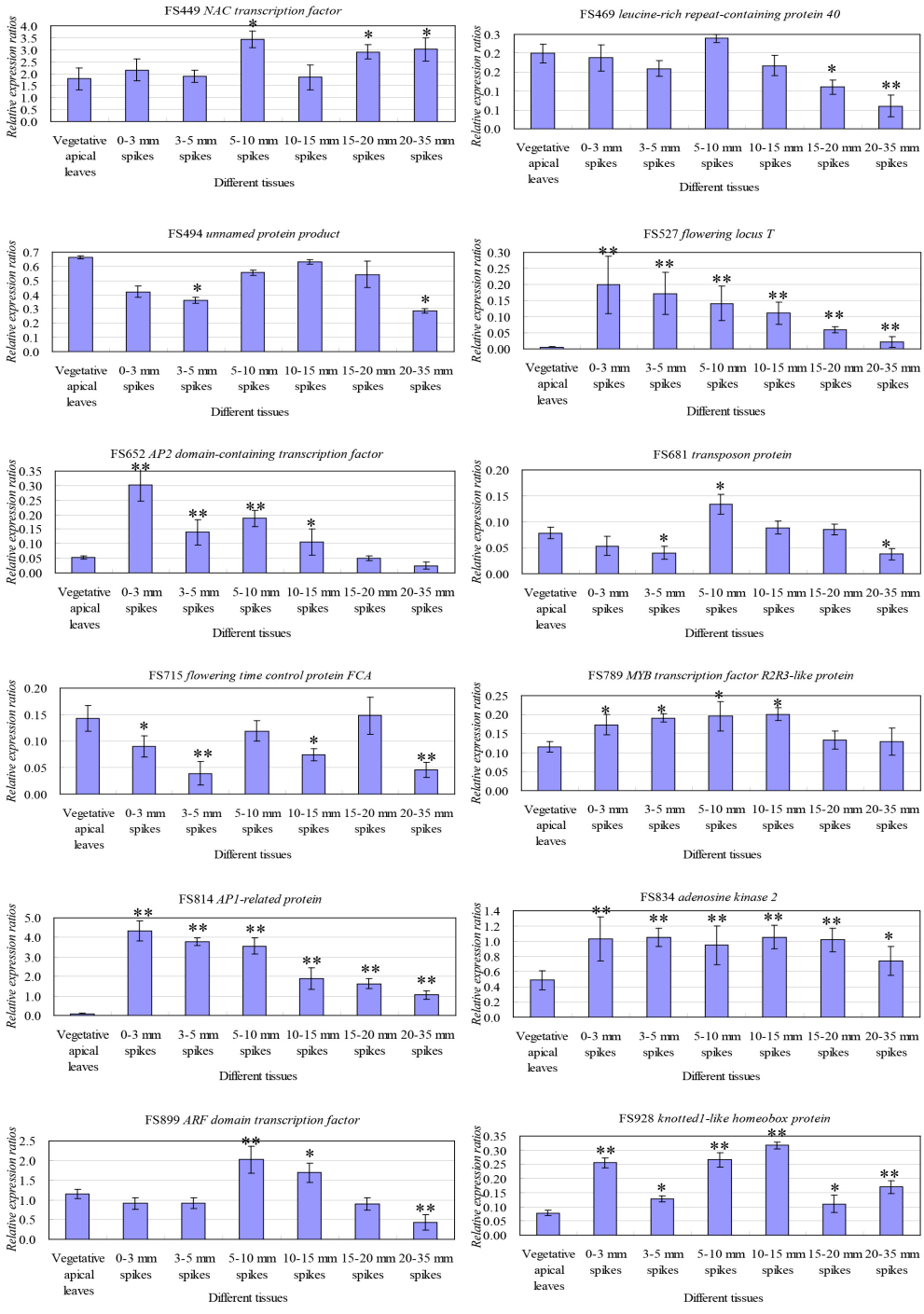
Figure 1. Continued.



In contrast, the genes downregulated in all spikes tested induced by warm day and cool night conditions were mainly involved in transcription regulation (FS71, *retrotransposon protein Ty3*; FS210, *C3HC4-type RING finger*) and unknown (FS494, *unnamed protein*) (Figure 1).

DISCUSSION

In this study, we constructed one forward subtracted cDNA library enriched for genes in spikes of *Phalaenopsis* hybrid Fortune Saltzman induced by warm days and cool nights. Our primary data provided new insights to the molecular events of flowering initiation in *Phalaenopsis* orchids that were induced by warm day and cool night conditions. The molecular mechanisms of flowering initiation in *Phalaenopsis* orchids are currently unclear. For clarifying the molecular mechanisms of flowering initiation in *Phalaenopsis* orchids, it is important to find out the flowering-related genes during floral transition. The SSH technique applied in this study was effective. There were 450 identified and sequenced ESTs. Their expressions were induced, increased or declined in *Phalaenopsis* hybrid Fortune Saltzman spikes at different developmental stages under warm days and cool nights, compared with the levels in juvenile apical leaves under warm daily temperatures. The diverse function of regulated genes found in our study revealed the complexity and variety of pathways involved in warm day and cool night treatment response in *Phalaenopsis* hybrid Fortune Saltzman, as shown for other plants.

In the present study, a number of *Phalaenopsis* hybrid Fortune Saltzman ESTs are homologous to flowering-related genes in various plants, such as Chinese *Cymbidium* (Huang et al., 2012; Xiang et al., 2012), *Doritaenopsis* hybrid (Sun et al., 2012), etc. Other ESTs identified in this study show sequence similarities with genes encoding proteins related to signal transduction, transportation, transcription regulation, and metabolic processes. Furthermore, real-time PCR was used to study the expressions of 24 genes selected (with putative functions) derived from the sequenced ESTs (Figure 1). Among them, 21 genes were found to be up-regulated in warm day- and cool night-induced spikes at some stages, compared with the levels in apical leaves under warm daily temperatures, while expressions of 3 other genes slightly or remarkably decreased in induced spikes at all developmental stages. Thus, selected warm day and cool night conditions induced *Phalaenopsis* hybrid Fortune Saltzman genes possibly contributing to the floral transition that will be discussed in the following sections.

Flowering pathway-related genes

Flowering pathway-related genes were identified from our library by BLASTx searches as described above and 9 putative flowering genes were identified. They were *LHY* (*late elongated hypocotyl*) (FS750), *DNA J homolog* (FS348), *FCA* (FS715), *FVE* (FS445), *FT* (FS527), *AP1* (FS814), *AP2* (FS652), *KNOX1* (FS184), and *knotted-like homeobox protein* (FS928). Of these genes, the 3 genes (*LHY*, *DNA J homolog*, *FVE*) transcript levels in induced spikes and juvenile apical leaves were not confirmed by real-time PCR, as we could not design appropriate primers. In *A. thaliana*, *J3* was involved in flowering signals from several genetic pathways and promoted flowering through directly activating the transcription of *SOC1* and *FT* during the floral transition (Shen et al., 2011); *LHY* participated in the photoperiodic control of flowering (Schaffer et al., 1998); *FVE* was involved in autonomous and cold response pathways regulating flowering time (Ausín et al., 2004; Jeon and Kim, 2011); *FCA* controlled

flowering by fluctuating ambient conditions and negative autoregulation pathways (Macknight et al., 1997; Quesada et al., 2003; Jung et al., 2012). In *Doritaenopsis* hybrid, *DhFVE* was also involved in the regulation of flowering (Sun et al., 2012). In our study, *FT*, *AP1*, *AP2*, *KNOX1*, and *knotted-like homeobox protein* transcripts accumulated to higher levels and *FCA* transcripts accumulated to lower levels in spikes of different sizes (0-15 mm) relative to juvenile apical leaves (Figure 1). In addition, expression patterns of *FT* and *AP1* were positively correlated with each other by real-time PCR in different developmental spikes (Figure 1). On the basis of the above findings, it is tempting to speculate that spikes of *Phalaenopsis* hybrid Fortune Saltzman induced by warm days and cool nights were at least related to 4 flowering genetic pathways: fluctuating ambient temperature, cold response, photoperiodic, and autonomous pathways. Future study is required to elucidate the function of these genes in *Phalaenopsis* hybrid Fortune Saltzman in the molecular flowering network.

Genes involved in metabolism

During floral transition induced by warm day and cool night conditions, expression of various *Phalaenopsis* hybrid Fortune Saltzman genes were involved in metabolism and biosynthesis of carbohydrates, amino acids, fatty acids, and secondary substances. S-adenosylmethionine synthase (SAMS) plays a regulatory role in the synthesis of methionine and other aspartate-derived amino acids (Tabor and Tabor, 1984). In the present study, the remarkably upregulated transcripts of *SAMS* in 0-5- and 10-25-mm spikes were relative to juvenile apical leaves (Figure 1). The results suggested that early differentiation and later developmental stages of spikes enhanced activity of amino acid metabolism, giving rise to more methionine and other aspartate-derived amino acids. In addition, we also found slightly or significantly increased levels of transcripts encoding NAD-dependent malate dehydrogenase (FS82), caffeoyl-CoA 3-O methyltransferase (FS276), dihydroflavonol 4-reductase (FS278), naringenin 3-dioxygenase (FS327), cytochrome P450 (FS383), and sesquiterpene synthase (FS397) in spikes of some stages relative to juvenile apical leaves. Expression regulation mechanisms of these genes in floral transition will be investigated in future studies.

Genes involved in transcriptional regulation

Transcription factors mediate regulation and expression of genes during diverse processes of plant life cycle. In warm day- and cool night-induced spikes, specific transcription factors encoding NAC, MYB and ARF proteins were obtained. These proteins also participated in flower organ development (Sablowski and Meyerowitz, 1998), amino acid metabolism (El-Kereamy et al., 2012), flavonoid biosynthesis (Nakatsuka et al., 2012), and red fruit-flesh phenotypes (Chagné et al., 2013), as well as response to different biotic and abiotic stresses (El-Kereamy et al., 2012; Li et al., 2012b). Compared with the expression levels in juvenile apical leaves, the accumulated transcripts of *NAC* (FS449) and *R2R3-like MYB* (FS789) were higher in all spikes tested, while the accumulated transcripts of *ARF* (FS899) were lower in early and later stages of spikes (Figure 1). These results indicated that *NAC* and *R2R3-like MYB* may positively regulate spike differentiation and development, and *ARF* may negatively regulate spike early differentiation and later developmental stages.

In this study, some regulators were also obtained, such as *retrotransposon protein*,

transposon protein and *zinc finger protein*. The accumulated transcripts of genes encoding retrotransposon protein Ty3 (FS71) and C_3HC_4 type ring finger (FS210) in different sizes of spikes were lower than in juvenile apical leaves. In addition, the gene (FS681) encoding transposon protein accumulated lower levels in spikes' early differentiation and later developmental stages than in juvenile apical leaves (Figure 1). These findings indicated that the 3 genes may negatively regulate spike early differentiation and later developmental stages. Future study is required to elucidate the functions of these genes in spike differentiation and development in *Phalaenopsis* hybrid Fortune Saltzman.

Genes involved in signal transduction

Genes of *Phalaenopsis* hybrid Fortune Saltzman involved in signal transduction during spike differentiation and development were also identified in this study. Examples of this category are genes encoding a protein kinase, a serine-threonine protein kinase, a glycogen synthase kinase, a signal recognition particle protein, an adenosine kinase and an SKP1/ASK1-like protein. The function of these genes in *Phalaenopsis* hybrid Fortune Saltzman remains to be elucidated. In the current study, the accumulated higher transcripts of *adenosine kinase 2* in spikes relative to juvenile apical leaves indicated the involvement of signaling events in response to warm day and cool night conditions. In fact, the *SKP1* homolog *ASK1* regulated development and interacted with the *UFO* gene to control floral organ identity in *A. thaliana* (Zhao et al., 1999). Moreover, *SKP1* was also involved in abscisic acid signaling to regulate seed germination, stomatal opening and root growth in *A. thaliana* (Li et al., 2012a).

Other genes

In the processes of spike differentiation and development induced by warm day and cool night conditions, expression of many genes known to be involved in defense- and stress-associated reactions, transportation, energy, and cell wall seems to be induced. Genes belonging to these categories include genes encoding heat shock protein, peroxidase, cold-acclimation specific protein, lectin, potassium channel, sugar transporter, ATPase, hydrolase, oxidoreductase, and cellulose. The function of these genes in *Phalaenopsis* hybrid Fortune Saltzman remains to be validated.

Finally, our study found that an unexpected high number of *Phalaenopsis* hybrid Fortune Saltzman ESTs corresponded to novel genes or putative proteins that had no determined function. It is interesting and of great importance to examine the transcript levels of these genes to gain insights into the molecular functions of these genes in spike differentiation and development induction by warm day and cool night conditions.

In summary, the present study indicated that the SSH technique could provide valuable insights into the expression patterns of genes associated with spike differentiation and development induction by warm day and cool night conditions in *Phalaenopsis* orchids. Sequence analysis of the library resulted in 450 *Phalaenopsis* hybrid Fortune Saltzman ESTs. On the basis of real-time PCR analysis of the selected genes, the validity of the library with respect to warm day- and cool night-induced expression was confirmed. Corresponding genes were predicted to encode proteins with various functions, suggesting the complex molecular alterations occurring in spike differentiation and development induction by warm days and cool nights in *Phalaenopsis* plants. Some of the identified ESTs were homologous to plant

flowering genes related to several flowering genetic pathways. Moreover, 22.22% of the warm day- and cool night-induced ESTs in spikes matched genes with unknown function in the NCBI database. Furthermore, we have cloned the full-length cDNAs of several genes (e.g., *FT*, *NAC*, and *R2R3-like MYB*) that could play important roles in spike differentiation and development induced by warm days and cool nights. The characterization of their functions is being undertaken to determine whether these warm day- and cool night-responsive genes have a role in flowering-time control. Such research effort will provide further insights into molecular mechanisms of floral transition induction by warm days and cool nights in *Phalaenopsis* orchids.

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REFERENCES

Abe M, Kobayashi Y, Yamamoto S, Daimon Y, et al. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator *FT* at the shoot apex. *Science* 309: 1052-1056.

Altschul SF, Madden TL, Schaffer AA, Zhang J, et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389-3402.

An FM, Hsiao SR and Chan MT (2011). Sequencing-based approaches reveal low ambient temperature-responsive and tissue-specific microRNAs in *Phalaenopsis* orchid. *PLoS One* 6: e18937.

Ausín I, Alonso-Blanco C, Jarillo JA, Ruiz-Garcia L, et al. (2004). Regulation of flowering time by FVE, a retinoblastoma-associated protein. *Nat. Genet.* 36: 162-166.

Chagné D, Lin-Wang K, Espley RV, Volz RK, et al. (2013). An ancient duplication of apple MYB transcription factors is responsible for novel red fruit-flesh phenotypes. *Plant Physiol.* 161: 225-239.

Chen D, Guo B, Hexige S, Zhang T, et al. (2007). SQUA-like genes in the orchid *Phalaenopsis* are expressed in both vegetative and reproductive tissues. *Planta* 226: 369-380.

Chen WH, Tseng YC, Liu YC, Chuo CM, et al. (2008). Cool-night temperature induces spike emergence and affects photosynthetic efficiency and metabolizable carbohydrate and organic acid pools in *Phalaenopsis aphrodite*. *Plant Cell Rep.* 27: 1667-1675.

Chen WS, Liu HY, Liu ZH and Yang L (1994). Gibberellin and temperature influence carbohydrate content and flowering in *Phalaenopsis*. *Physiol. Plantarum* 90: 391-395.

Chen YH, Tsai YJ, Huang JZ and Chen FC (2005). Transcription analysis of peloric mutants of *Phalaenopsis* orchids derived from tissue culture. *Cell Res.* 15: 639-657.

Corbesier L, Vincent C, Jang S, Fornara F, et al. (2007). FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. *Science* 316: 1030-1033.

El-Kereamy A, Bi YM, Ranathunge K, Beatty PH, et al. (2012). The rice R2R3-MYB transcription factor *OsMYB55* is involved in the tolerance to high temperature and modulates amino acid metabolism. *PLoS One* 7: e52030.

Endo M and Ikusima I (1989). Diurnal rhythm and characteristics of photosynthesis and respiration in the leaf and root of a *Phalaenopsis* plant. *Plant Cell Physiol.* 30: 43-47.

Guo WJ and Lee N (2006). Effect of leaf and plant age, and day/night temperature on net CO_2 uptake in *Phalaenopsis amabilis* var. Formosa. *J. Am. Soc. Hort. Sci.* 131: 320-326.

Hew CS and Yong JWH (1997). Physiology of Tropical Orchids in Relation to the Industry. World Scientific, Singapore.

Hsiao YY, Tsai WC, Kuoh CS, Huang TH, et al. (2006). Comparison of transcripts in *Phalaenopsis bellina* and *Phalaenopsis equestris* (Orchidaceae) flowers to deduce monoterpene biosynthesis pathway. *BMC Plant Biol.* 6: 14.

Hsiao YY, Chen YW, Huang SC, Pan ZJ, et al. (2011). Gene discovery using next-generation pyrosequencing to develop ESTs for *Phalaenopsis* orchids. *BMC Genomics* 12: 360.

Huang W, Fang Z, Zeng S, Zhang J, et al. (2012). Molecular Cloning and Functional Analysis of Three *FLOWERING LOCUS* T (FT) Homologous Genes from Chinese *Cymbidium*. *Int. J. Mol. Sci.* 13: 11385-11398.

Jeon J and Kim J (2011). FVE, an *Arabidopsis* homologue of the retinoblastoma-associated protein that regulates flowering time and cold response, binds to chromatin as a large multiprotein complex. *Mol. Cells* 32: 227-234.

Jung JH, Seo PJ, Ahn JH and Park CM (2012). *Arabidopsis* RNA-binding protein FCA regulates microRNA172 processing in thermosensory flowering. *J. Biol. Chem.* 287: 16007-16016.

Lee J and Lee I (2010). Regulation and function of SOC1, a flowering pathway integrator. *J. Exp. Bot.* 61: 2247-2254.

Lee J, Oh M, Park H and Lee I (2008). SOC1 translocated to the nucleus by interaction with AGL24 directly regulates *LEAFY*. *Plant J.* 55: 832-843.

Li C, Liu Z, Zhang Q, Wang R, et al. (2012a). SKP1 is involved in abscisic acid signalling to regulate seed germination, stomatal opening and root growth in *Arabidopsis thaliana*. *Plant Cell Environ.* 35: 952-965.

Li DM, Wang JH, Peng SL, Zhu GF, et al. (2012b). Molecular cloning and characterization of two novel NAC genes from *Mikania micrantha* (Asteraceae). *Genet. Mol. Res.* 11: 4383-4401.

Macknight R, Bancroft I, Page T, Lister C, et al. (1997). *FCA*, a gene controlling flowering time in *Arabidopsis*, encodes a protein containing RNA-binding domains. *Cell* 89: 737-745.

Nakatsuka T, Saito M, Yamada E, Fujita K, et al. (2012). Isolation and characterization of *GtMYBP3* and *GtMYBP4*, orthologues of R2R3-MYB transcription factors that regulate early flavonoid biosynthesis, in *gentian* flowers. *J. Exp. Bot.* 63: 6505-6517.

Pollet B, Kromwijk A, Vanhaecke L, Dambre P, et al. (2011a). A new method to determine the energy saving night temperature for vegetative growth of *Phalaenopsis*. *Ann. Appl. Biol.* 158: 331-345.

Pollet B, Vanhaecke L, Dambre P, Lootens P, et al. (2011b). Low night temperature acclimation of *Phalaenopsis*. *Plant Cell Rep.* 30: 1125-1134.

Quesada V, Macknight R, Dean C and Simpson GG (2003). Autoregulation of FCA pre-mRNA processing controls *Arabidopsis* flowering time. *EMBO J.* 22: 3142-3152.

Sablowski RW and Meyerowitz EM (1998). A homolog of NO APICAL MERISTEM is an immediate target of the floral homeotic genes APETALA3/PISTILLATA. *Cell* 92: 93-103.

Sakanishi Y, Imanishi H and Ishida G (1980). Effect of temperature on growth and flowering of *Phalaenopsis amabilis*. *Bull. Univ. Osaka Pref. Ser. B* 32: 1-9.

Schaffer R, Ramsay N, Samach A, Corden S, et al. (1998). The late elongated hypocotyl mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. *Cell* 93: 1219-1229.

Shen L, Kang YG, Liu L and Yu H (2011). The J-domain protein J3 mediates the integration of flowering signals in *Arabidopsis*. *Plant Cell* 23: 499-514.

Srikanth A and Schmid M (2011). Regulation of flowering time: all roads lead to Rome. *Cell Mol. Life Sci.* 68: 2013-2037.

Sun X, Qin Q, Zhang J, Zhang C, et al. (2012). Isolation and characterization of the FVE gene of a *Doritaenopsis* hybrid involved in the regulation of flowering. *Plant Growth Regul.* 68: 77-86.

Tabor CW and Tabor H (1984). Methionine adenosyltransferase (S-adenosylmethionine synthetase) and S-adenosylmethionine decarboxylase. *Adv. Enzymol. Relat. Areas Mol. Biol.* 56: 251-282.

Tsai WC, Hsiao YY, Lee SH, Tung CW, et al. (2006). Expression analysis of the ESTs derived from the flower buds of *Phalaenopsis equestris*. *Plant Sci.* 170: 426-432.

Wang YT (1995). *Phalaenopsis* orchid light requirement during the induction of spiking. *Hort. Sci.* 30: 59-61.

Xiang L, Li X, Qin D, Guo F, et al. (2012). Functional analysis of *FLOWERING LOCUS* T orthologs from spring orchid (*Cymbidium goeringii* Rchb. f.) that regulates the vegetative to reproductive transition. *Plant Physiol. Biochem.* 58: 98-105.

Zhang JX, Wu KL, Tian LN, Zeng SJ, et al. (2011). Coning and characterization of a novel CONSTANS-like gene from *Phalaenopsis* hybrida. *Acta Physiol. Plant.* 33: 409-417.

Zhao D, Yang M, Solava J and Ma H (1999). The ASK1 gene regulates development and interacts with the UFO gene to control floral organ identity in *Arabidopsis*. *Dev. Genet.* 25: 209-223.