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ORIGINAL PAPER

Transcriptomic analysis of floral initiation in litchi (*Litchi chinensis* Sonn.) based on de novo RNA sequencing

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Abstract

Key message **Comparative transcriptome analysis of litchi (*Litchi chinensis* Sonn.) buds at two developmental stages revealed multiple processes involving various phytohormones regulating floral initiation, and expression of numerous flowering-related genes.**

Abstract Floral initiation is a critical and complicated plant developmental process involving interactions of numerous endogenous and environmental factors, but little is known about the complex network regulating floral initiation in litchi (*Litchi chinensis* Sonn.). Illumina second-generation sequencing is an efficient method for obtaining massive transcriptional information resulting from phase changes in plant development. In this study, comparative transcriptomic analysis was performed with resting and emerging panicle stage buds, to gain further understanding of the molecular mechanisms involved in floral initiation in litchi. Abundance analysis identified 5,928 unigenes exhibiting at least twofold differences in expression between the two bud stages. Of these, 4,622 unigenes were up-regulated and 1,306 were down-regulated in panicle-emerging buds compared with resting buds. KEGG pathway enrichment analysis revealed that unigenes exhibiting differential expression were involved in the metabolism and signal transduction of various phytohormones. The expression levels of unigenes annotated as auxin, cytokinin, jasmonic acid, and salicylic acid biosynthesis were up-regulated, whereas those unigenes annotated as abscisic acid biosynthesis were down-regulated during floral initiation. In addition, 188 unigenes exhibiting sequence similarities to known flowering-related genes from other plants were differentially expressed during floral initiation. Thirteen genes were selected for confirmation of expression levels using quantitative-PCR. Our results provide abundant sequence resources for studying mechanisms underlying floral initiation in litchi and establish a platform for further studies of litchi and other evergreen fruit trees.

Keywords Floral initiation · Transcriptome · Phytohormone · Litchi (*Litchi chinensis* Sonn.)

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Introduction

Litchi (*Litchi chinensis* Sonn.), an evergreen fruit tree, is native to southern China and widely distributed in the subtropics. Irregularities in flowering resulted in fluctuating yield have caused strong concerns. In the northern hemisphere, most litchi varieties form flowers after winter chilling due to the requirements for low temperatures for floral induction and ascending temperatures for flower initiation and development. To respond to floral induction,

the terminal shoots of litchi must be mature. Then, the buds remain dormant until the temperature increases and water stress is released, and the apical buds undergo floral initiation (Chen 2002; Huang and Chen 2003). Either improper shoot state or warm or humid winter conditions may cause unreliable flowering in litchi (Chen et al. 2014).

Floral initiation, a critical phase switch during flower development, is controlled by a combination of endogenous and environmental signals that activate appropriate morphological programs. Considerable progresses have been made toward understanding the genetic pathways involved in floral initiation in the model plant *Arabidopsis* (Andres and Coupland 2012; Jack 2004; Krizek and Fletcher 2005). Light and temperature are the two important external factors that regulate flowering through the photoperiod and vernalization pathways, respectively. Environmental factors often affect plant development by stimulating changes in hormone levels, and studies are increasingly revealing the relationships between phytohormones and flowering (Davis 2009; Domagalska et al. 2010). Cytokinins (CKs) are believed to promote floral transition, as shown by the fact that *Arabidopsis* plants deficient in CKs or impaired in CK signaling were late flowering (Corbesier et al. 2003; Werner et al. 2003; Bartrina et al. 2011). CKs are able to activate *SaMADS A*, a gene apparently involved in the regulation of floral transition in *Sinapis alba* (Bonhomme et al. 2000). Endogenous cytokinin levels increased in buds of litchi at the onset of floral initiation and differentiation, and exogenous applications did promote floral initiation in litchi (Chen 1991). Abscisic acid (ABA) is considered to be a floral repressor and may bind to the Flowering control locus A (FCA) protein in the autonomous flowering pathway (Barrero et al. 2005; Razem et al. 2006). SA and JA have also been shown to be involved in the floral transition (Davies 2004). For example, SA-deficient Arabidopsis exhibited a late-flowering phenotype in a *FLC*-independent way under long-day condition (Martinez et al. 2004). During flower and seed development, JA content rose and expression of jasmonate-inducible genes increased (Wasternack et al. 2013). Thus, floral initiation in the apical meristem relies on multiple hormonal pathways, and the corresponding phytohormones were likely to impinge on the signal network leading to reproductive phase transition (Davis 2009). However, in the case of litchi, the lack of genetic background has become a limitation for extensive research on flower event.

The flowering transcriptome was analyzed in *Arabidopsis*, and a large number of flowering-related genes were identified (Hennig et al. 2004; Wellmer et al. 2006). cDNA microarrays were used to identify candidate genes expressed during flower development in rose (Dubois et al. 2011). Similarly, cDNA amplified fragment length polymorphism analysis and proteomic techniques were used to reveal many comprehensive molecular genetic events related to floral transition in *Agapanthus* (Zhang et al. 2013). Recently, deep sequencing has been widely used to acquire a global view of transcriptomic dynamics during developmental processes, such as flower development (Singh et al. 2013). Further, RNA-Seq analysis of *Dendrocalamus latiflorus* and soybean revealed major events that occur during floral transition (Wong et al. 2013; Zhang et al. 2012). For high heterozygous woody plants, especially those without whole genome database, transcriptome-wide analysis is an efficient method to obtain unigene information on how expression of unigenes contributes to the process.

In the present study, RNA samples from litchi buds at two developmental stages were sequenced using an Illumina HiSeq 2000. The objective was to obtain an understanding of molecular mechanisms in litchi flower initiation and establish a foundation for further study based on high-throughput sequencing.

Materials and methods

Plant materials

The samples were collected from the litchi trees growing in Wushan, Guangzhou, China. The floral phenology of 'Feizixiao' litchi trees was recorded according to the *Biologische Bundesanstalt*, *Bundessortenamt and Chemische Industrie* (BBCH)-scale (Wei et al. 2013). The air temperature was recorded continuously from September 2009 to May 2012 (Fig. 1b). The visible morphological changes during flower development, from the vegetative meristem stage to the full bloom stage, were observed (Fig. 1a). The autumnal terminal shoots reached a state of maturity before November and remained dormant in response to low temperatures during the winter. As temperature increased in early spring, apical buds swelled, elongated, and attained the floral initiation state in mid-January (Fig. 1b). The buds continued to develop into flower panicles and subsequently bloomed.

To examine molecular events related to floral initiation in litchi, buds at two stages were collected from 15 ten-year-old trees of cv. 'Feizixiao' in 2011/2012 season: resting buds in the later stage of floral induction (phase 1, December 20, 10–15 °C, Figs. 1a, 2, 3) and panicle-emerging buds during floral initiation (phase 2, February 10, 20–25 °C, Figs. 1a, 2, 3, 4), which represent the two phases of bud development before and after floral transition. Bud developmental stages were described (Fig. 1a), and resting buds in the floral induction phase (Figs. 1a, 2, 3) and panicle-emerging buds in the floral initiation phase (Figs. 1a, 2, 3, 4) were frozen immediately in liquid nitrogen and stored at −80 °C.

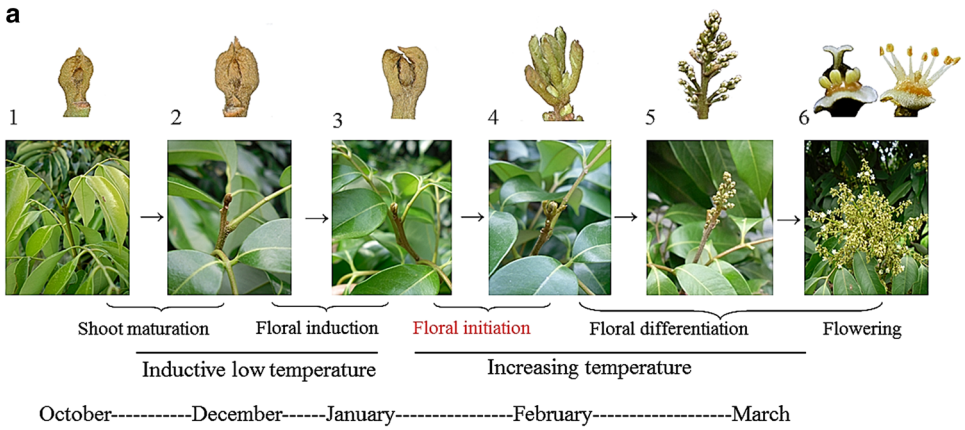

Fig. 1 Samples used in this work. **a** Schematic diagram of the developmental stages in flowering of litchi. Phase changes in floral formation in 'Feizixiao' litchi were observed. Phenology stages are numbered according to the BBCH scale adapted for 'Feizixiao' litchi trees (Wei et al. 2013). **b** Average daytime temperatures from September 1st 2009 to April 1st 2012. The time of floral initiation in 'Feizixiao' litchi is indicated with *red-dashed lines*

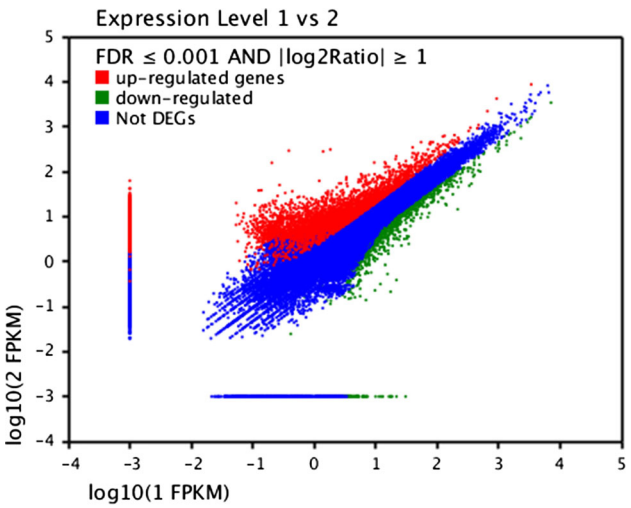

Fig. 2 Comparison of transcript abundance levels between phases 1 and 2. Differentially expressed sequences are indicated in *red* (increased expression) and *green* (decreased expression). *Blue* indicates sequences that were not differentially expressed (Color figure online)

RNA extraction and cDNA synthesis

RNA was extracted from entire buds, including floral meristems and their surrounding juvenile leaves. Total RNA was extracted using the Quick RNA Isolation Kit (Huayueyang, China) according to the manufacturer's instruction. DNase I (Takara, Japan) was added to remove genomic DNA, and RNase-free columns (Huayueyang, China) were used for total RNA purification (Wei et al. 2011). The integrity and quality of RNA were evaluated by agarose gel electrophoresis and the BioPhotometer Plus photometer (Eppendorf, Germany). cDNA was synthesized from 2 μg total RNA and oligo (dT) primers using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA).

Library preparation and sequencing

A schematic overview of library preparation and sequencing, including Illumina sequencing, data assembly, and bioinformatic analysis of the transcriptome, is presented in Fig. S1. The

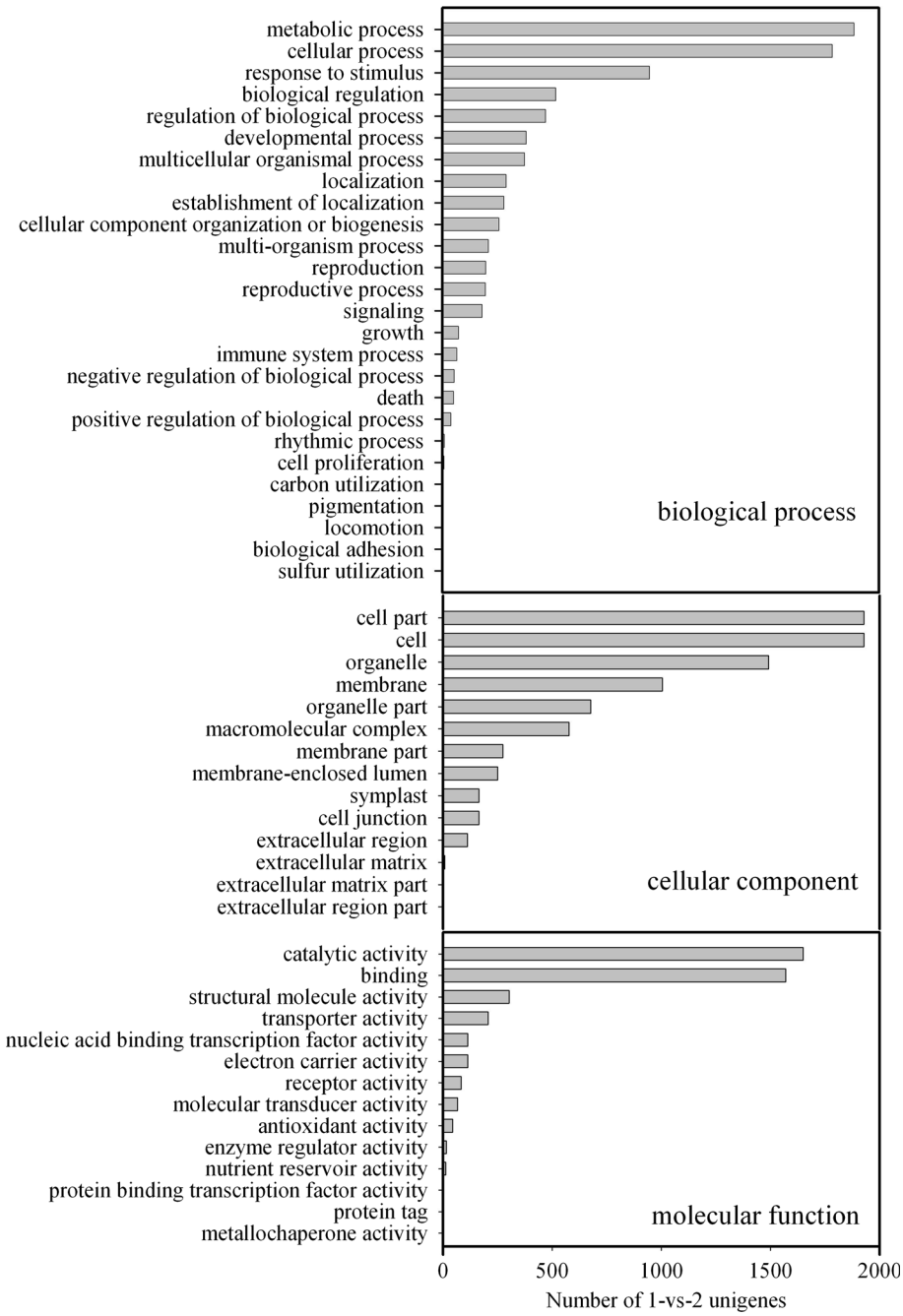

Fig. 3 GO classification of differentially expressed unigenes. Unigenes were assigned to three main categories: biological process, cellular components, and molecular function. Values are displayed for each term as the percent of the total number of genes as well as the number of genes

de novo sequencing workflow was reported earlier for *D. latiflorus* (Zhang et al. 2012). Library products were also sequenced using the HiSeq^{TM} 2000 platform (Illumina) and analyzed at Beijing Genomics Institute (Shenzhen).

Quantitative-PCR (q-PCR) analysis

The accuracy of the transcriptomic data was confirmed using q-PCR analysis of thirteen selected differentially expressed genes during floral transition in litchi. Gene-specific primers were designed using the Primer 5.0 program (PREMIER Biosoft International, Canada) (Table S1). Transcript levels were analyzed using q-PCR, with the DyNAmo Flash SYBR Green q-PCR kit (Thermo, USA) and the CFX96 q-PCR System (Bio-Rad, USA), according to the manufacturer's instructions. The reaction mixtures were heated to 95 °C for 7 min, followed by 38 cycles at 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 25 s. The melting curves were plotted to determine the specificity of the amplification reactions. All reactions in q-PCR were normalized using C_t values corresponding to *actin* levels, according to a study of reliable reference genes for expression study by q-PCR in litchi (Wei et al. 2011; Zhong et al. 2011). Unigene expression

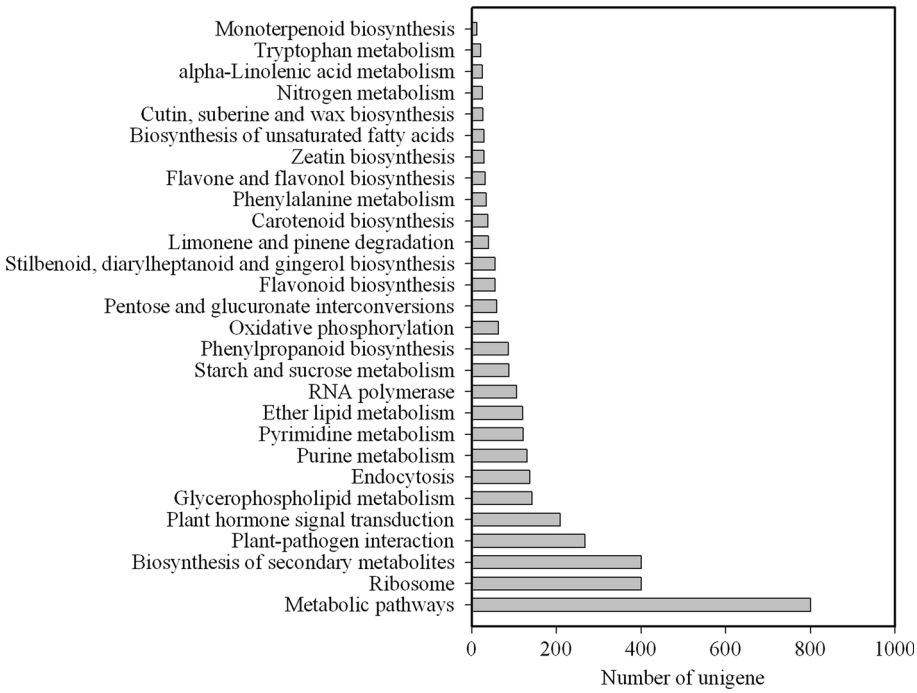

Fig. 4 KEGG pathways significantly enriched for differentially expressed unigenes. A total of 3,562 differentially expressed unigenes were significantly enriched in 28 KEGG pathways

Table 1 Summary statistics of the sequence assembly

	Phase 1	Phase 2
Total clean reads	41,516,696	44,950,238
Total clean nucleotides	3,736,502,640	4,045,521,420
Total number of contigs	90,759	112,756
Average contig length (bp)	372	331
Total number of unigenes	52,134	57,334
Average unigene length (bp)	687	669

levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). Values of each time point were the averages of three technical replicates of each biological replicate.

Results

cDNA sequence synthesis and de novo assembly

RNA isolated from samples was subjected to reverse transcription, and cDNA was sequenced using an Illumina HiSeq 2000. The processes for bioinformatic analysis of the RNA-Seq data are listed in Fig. S1. After cleaning and quality checks, approximately 87 million reads (about 42 million and 45 million for phases 1 and 2, respectively) were obtained from the RNA-Seq data (Table 1). Assembly of reads resulted in 90,759 and 112,756 contigs with mean sizes of 372 and 331 bp for phases 1 and 2, respectively. Using paired end-joining and gap-filling methods, these contigs were further assembled into 52,134 putative unigenes with an average length of 687 bp for phase 1, and 57,334 unigenes with an average length of 669 bp for phase 2 (Table 1). The size distributions of the transcriptome assembly are presented in Fig. S2. Assembled unigenes from the two phases were used for sequence splicing and redundancy removal with sequence clustering software, and non-redundant 53,453 unigenes were acquired.

Functional annotation of the litchi flower transcriptome

A BLASTx (E value $<10^{-5}$) search using public protein and nucleotide databases [Nr, Nt, Gene Ontology (GO), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Clusters of Orthologous Groups (COG)] was performed with sequences from 53,453 unigenes. Approximately, 41,347 (77.35 %) unigenes were successfully annotated in this analysis and are listed in Table S2. Of these annotated genes, 39,892 (74.63 %) had matches to the Nr database. The overall summary of functional annotation is presented in Table S3. According to the E value distribution of the top hits from the Nr database, 48.6 % of the sequence matches had strong sequence similarities (E value $<10^{-60}$) and 51.4 % had moderate sequence similarities ($10^{-60} < E$ value $< 10^{-5}$) (Fig. S3a). The similarity distribution pattern indicated that 27.1 % of the sequences had a similarity higher than 80 %, whereas 72.9 % had a similarity between 18 and 80 % (Fig. S3b). 30 % of the unique sequences had top matches to sequences from *Vitis vinifera*, 25.2 % to *Ricinus communis*, 21.9 % to *Populus trichocarpa*, 5.6 % to *Glycine max*, and 2.0 % to *Medicago truncatula* (Fig. S3c).

To identify candidate genes involved in floral initiation, we performed differential expression analysis with the two samples. A total of 5,928 unigenes showed at least a twofold difference in expression between the two phases. Among these, 4,622 unigenes were up-regulated in phase 2 compared with phase 1 and likely to represent activators associated with floral initiation, whereas 1,306 unigenes were down-regulated in phase 2 and likely to represent potential repressors associated with floral initiation (Fig. 2; Table S4).

GO annotation

All differentially expressed unigenes between phases 1 and 2 were subjected to GO analysis, and 21,081 were assigned GO terms (Fig. 3; Table S5). For the biological process category, "cellular process" and "metabolic processes" were the most frequently assigned GO terms. For molecular functions, the unigenes were assigned primarily to "binding" and "catalytic activity". For the cellular component category, a large number of genes were assigned to "cell part" and "cell".

COG annotation

All unigenes were searched against the COG database to predict and classify their possible functions. Overall, 29,871 sequences were classified into 25 COG categories (Fig. S4; Table S6). The majority of the unigenes was clustered into categories for general function only (4,917; 16.46 %); transcription (2,832; 9.48 %); replication, recombination, and repair (2,385; 7.98 %); translation, ribosomal structure, and biogenesis (2,233; 7.48 %); post-translational modification, protein turn over, and chaperones (2,180; 7.30 %); signal transduction mechanisms (1,960, 6.56 %); and carbohydrate transport and metabolism (1,785; 5.98 %). The smallest clusters were extracellular and nuclear structures (14 and 3 unigenes, respectively).

KEGG pathway mapping

Pathway-based analysis can help us to further understand the biological functions of genes. The KEGG pathway database contains plenty of information on networks of intracellular molecular interactions, and their organism-specific variations (Kanehisa et al. 2008). To better understand the functions of sequenced genes in litchi, we mapped the annotated sequences to the reference standard pathways contained in the KEGG database. Referencing 23,669 unigenes through the KEGG database predicted 123 pathways (Table S7), representing compound biosynthesis, degradation, and utilization. Some predictions were consistent with the results of GO assignment. After KEGG enrichment analysis, 3,562 differentially expressed unigenes were significantly enriched in 28 pathways ($P \leq 0.05$, Fig. 4). The most represented were metabolic pathways (801; 22.4 %), ribosome (401; 11.3 %), and biosynthesis of secondary metabolites (400; 11.2 %). Plant–pathogen interaction (268; 7.5 %) and plant hormone signal transduction (209; 5.8 %) pathways were also well represented (Fig. 4; Table S7). Genes involved in pathways responsible for carbohydrate metabolism, such as starch, sucrose, as well as pentose and glucuronate interconversion pathways, were also differentially expressed. Lipid metabolism pathways, including metabolisms of glycerophospholipid, ether lipid, unsaturated fatty acid, cutin, suberin and wax, were enriched. Pathways involved in amino acid metabolisms, such as phenylpropanoid and tryptophan metabolism, were also enriched. In addition, pathways related to phytohormone biosynthesis, such as zeatin biosynthesis, tryptophan metabolism, alpha-linolenic acid metabolism, phenylalanine metabolism, and carotenoid biosynthesis, were well represented.

Phytohormone events associated with floral initiation

Phytohormone levels have been reported to be closely correlated with the transition and development of flowers (Blazquez et al. 2006; Heisler et al. 2005). To investigate the relationship between phytohormones and floral transition in litchi, the enriched phytohormone signal transduction pathways and the KEGG pathways related to some phytohormone biosynthesis were analyzed.

Differential expression of ABA metabolism-related genes was observed by analyzing the carotenoid metabolism pathway (Fig. 4; Fig. S5a). Transcript abundance of four unigenes in the ABA biosynthesis pathway, which were annotated as 9-cisepoxy *carotenoid dioxygenase* (*NCED*) and *carotenoid cleavage dioxygenase 4* (*CCD*), decreased in flower buds in phase 2. Conversely, six unigenes annotated as *ABA 8′-hydroxylase* (*CYP707A*), the key enzyme for ABA degradation (Kushiro et al. 2004), were more abundant in phase 2 (Table 2). These results suggested that the ABA level might decrease towards floral initiation.

Auxin metabolism is associated with the tryptophan metabolic pathway that was highly enriched in the KEGG pathways (Fig. 4; Fig. S5b). Transcript abundance of six unigenes in the auxin biosynthesis pathway, annotated as *tryptophan synthase*, *indole-3-acetic acid* (*IAA*)-*amino acid hydrolase*, and *YUCCA*, increased in phase 2 (Table 2). Differential expression of unigenes related to CK metabolism was observed by analyzing the enriched zeatin biosynthesis pathway. Transcripts of genes annotated as *triosephosphate isomerase*, *Cytokinin oxidase,* and

Table 2 List of differentially expressed genes related to phytohormone biosynthesis during floral initiation

Gene ID	Phase 1 (RPKM)	Phase 2 (RPKM)	Log 2 (phase 2/phase 1)	Annotation
ABA				
Unigene19450_All	359.61	53.59	−2.75	9-*cis*-epoxycarotenoid dioxygenase (NCED) [*Citrus sinensis*]
Unigene23025_All	5.19	0.98	−2.40	9-*cis*-epoxycarotenoid dioxygenase (NCED) [*Ricinus communis*]
Unigene22240_All	3.25	0.77	−2.08	Carotenoid cleavage dioxygenase 4 (CCD) [*Osmanthus fragrans*]
Unigene25154_All	2.37	1.12	−1.08	Carotenoid cleavage dioxygenase 4 (CCD) [*Citrus clementina*]
Unigene17907_All	1.28	12.90	3.33	ABA 8′-hydroxylase (CYP707A) [*Citrus sinensis*]
Unigene19707_All	1.06	8.67	3.04	ABA 8′-hydroxylase (CYP707A) [*Vitis vinifera*]
Unigene29475_All	0.12	3.19	4.73	ABA 8′-hydroxylase (CYP707A) [*Citrus sinensis*]
Unigene29476_All	0.00	3.71	11.86	ABA 8′-hydroxylase (CYP707A) [*Vitis vinifera*]
Unigene30544_All	1.06	6.01	2.51	ABA 8′-hydroxylase (CYP707A) [*Citrus sinensis*]
Unigene30678_All	0.42	4.63	3.45	ABA 8′-hydroxylase (CYP707A) [*Citrus sinensis*]
Auxin				
Unigene16401_All	66.12	244.69	1.89	Tryptophan synthase [*Vitis vinifera*]
Unigene30656_All	1.11	4.38	1.98	IAA-amino acid hydrolase [*Populus trichocarpa*]
Unigene20451_All	66.53	184.41	1.47	IAA-amino acid hydrolase [*Ricinus communis*]
CL6910.Contig3_All	29.74	84.58	1.51	IAA-amino acid hydrolase [*Vitis vinifera*]
Unigene29386_All	0.00	3.01	11.55	YUCCA [Populus trichocarpa]
CL487.Contig2_All	9.53	32.87	1.79	YUCCA [*Populus trichocarpa*]
CK				
Unigene26638_All	0.12	4.89	5.38	Triosephosphateisomerase [*Zea mays*]
Unigene21026_All	2.51	5.76	1.20	Cytokinin oxidase [*Populus trichocarpa*]
CL6176.Contig1_All	0.50	5.56	3.48	Gulonolactone oxidase [*Ricinus communis*]
Unigene287_All	1.82	22.14	3.61	Gulonolactone oxidase [*Ricinus communis*]
JA				
CL4348.Contig1_All	28.88	72.16	1.32	Linoleate 13S-lipoxygenase [*Populus trichocarpa*]
CL4952.Contig1_All	6.16	1.41	−2.13	Jasmonate-*O*-methyltransferase [*Ricinus communis*]
SA				
CL4886.Contig5_All	145.28	38.59	−1.91	Phenylalanine ammonia-lyase (PAL) [*Camellia sinensis*]
CL4886.Contig6_All	19.10	10.61	−0.85	Phenylalanine ammonia-lyase (PAL) [*Citrus limon*]
Unigene6356_All	9.80	5.32	−0.88	Phenylalanine ammonia-lyase (PAL) [*Vitis vinifera*]
Unigene24017_All	6.37	2.32	−1.46	Phenylalanine ammonia-lyase (PAL) [*Litchi chinensis*]
Unigene23789_All	79.65	30.21	−1.40	Phenylalanine ammonia-lyase (PAL) [*Ricinus communis*]
Unigene30790_All	66.81	27.47	−1.28	Phenylalanine ammonia-lyase (PAL) [*Ricinus communis*]
CL4886.Contig1_All	1.24	0.55	−1.19	Phenylalanine ammonia-lyase (PAL) [*Populus trichocarpa*]
CL4886.Contig2_All	26.77	12.57	−1.09	Phenylalanine ammonia-lyase (PAL) [*Litchi chinensis*]
CL4886.Contig3_All	4.76	1.78	−1.42	Phenylalanine ammonia-lyase (PAL) [*Litchi chinensis*]
CL4886.Contig4_All	73.2972	31.633	−1.21	Phenylalanine ammonia-lyase (PAL) [*Litchi chinensis*]

gulonolactone oxidase were markedly more abundant in phase 2 (Table 2). An overall increase in transcripts related to CK biosynthesis implies an increase in CK levels during floral initiation.

Moreover, differential expression of genes involved in other phytohormone metabolic pathways was also identified (Table 2). JA and SA biosynthesis pathways are associated with alpha-linolenic acid metabolism and phenylalanine biosynthesis, both of which were highly enriched in KEGG pathways (Fig. 4; Fig. S5d, e). Genes encoding JA biosynthesis enzymes such as *linoleate 13S-lipoxygenase* were up-regulated, whereas *jasmonate-O-methyltransferase*, whose product deactivates jasmonate, was down-regulated in phase 2 (Table 2). Transcripts of 10 genes encoding phenylalanine ammonia-lyase (PAL), a key enzyme for SA biosynthesis, were more abundant in phase 2 (Table 2). These results suggested that an increase in jasmonates and salicylic acid levels might occur towards

floral initiation. However, the roles of jasmonates and salicylic acid in floral initiation are still not well understood.

A total of 62 differentially expressed unigenes were related to the plant hormone signal transduction pathways (Table S8; Fig. S5f), among which ABA, auxin, CK, JA and SA signaling pathways were further analyzed (Fig. 5). Eight unigenes annotated as *PROTEIN PHOSPHATASE 2C* (*PP2C*) involved in ABA signal transduction were differentially expressed, and seven out of the eight were up-regulated in phase 2, five genes annotated as ABA-responsive element-binding factor (ABF) were down-regulated (Fig. 5a). In the auxin-responsive pathway, two genes annotated as *auxin response factors* (*ARFs*) were up-regulated, whereas one auxin receptor *transport inhibitor response 1* (*TIR1*) gene was down-regulated in phase 2 (Fig. 5c). In addition, the three families of early auxin-responsive genes, Aux/IAA (1), GH3 (3), and SAUR (1), were up-regulated during floral initiation (Fig. 5a). Of the unigenes related to CK signaling, the levels of three *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) genes, annotated as *ARR8* and *ARR17*, increased in phase 2 (Fig. 5e). In the cytokinin-responsive pathway, two genes encoding *transmembrane histidine kinases* (*HK4*), which are cytokinin receptors, were found to be up-regulated during floral initiation (Fig. 5e). Floral initiation also appeared to up-regulate expressions of JA- and SA-related genes. Upregulation of three *Jasmonates ZIM-domain protein* (*JAZ*) genes and one *MYC2* gene occurs during floral initiation (Fig. 5d). Similarly, in the SA signaling pathway, the levels of three unigenes annotated as *pathogenesis-related protein 1a (PR-1)* and *non-expressor of PR1 (NPR1)*, increased in phase 2, whereas the unigene annotated as *BZIP transcription factor family protein* (*TGA*) decreased in phase 2 (Fig. 5b). Several genes related to the hormone signaling pathway were also selected for q-PCR analysis. Levels of *Aux/IAA* and *ARF* (auxin-responsive genes), *ARR8* (a positive regulator of CK), and *JAZ* (a positive regulator of JA) increased in phase 2, which exhibited similar expression patterns with RNA-Seq results (Fig. 6).

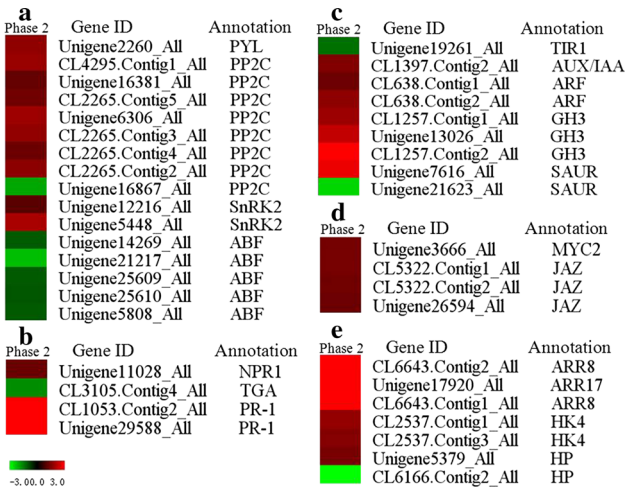

Fig. 5 Heat map diagrams of relative expression levels of differentially expressed unigenes annotated as genes in phytohormone signal transduction pathways identified in KEGG pathway enrichment analysis. The differentially expressed unigenes annotated as genes in the signal transduction pathways of ABA (**a**), SA (**b**), auxin (**c**), JA (**d**), and CKs (**e**) are shown. All differentially expressed unigenes within the phytohormone signal transduction pathways are listed in Table S8. The ratios were expressed as $\log_2$ RPKM (phase 2/phase 1) values and differential expression was indicated by a color scale based on the coefficient of variation: *Red* and *green colors* indicate up and downregulation of genes in phase 2 (Color figure online)

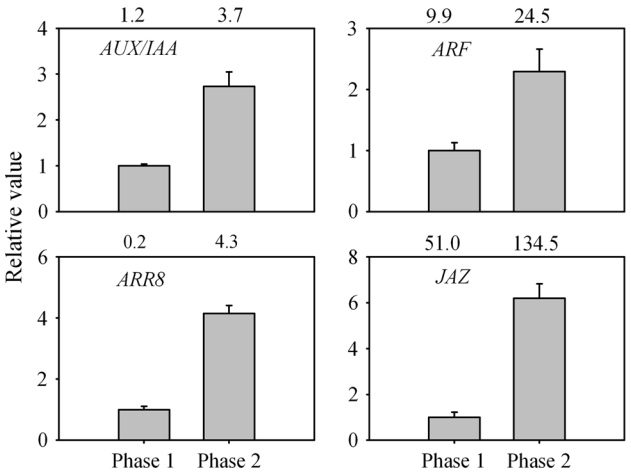

Fig. 6 Real-time quantitative expression of four genes involved in plant hormone signal transduction pathways during floral initiation. The RPKM value of two samples from RNA-Seq data is listed above each gene. Relative transcript levels are calculated by q-PCR using β-actin as a standard. *Error bars* indicate the standard deviation of three technical replicates

Identification of flowering-related genes expressed during floral initiation

Molecular and genetic regulation of flower development has been extensively investigated in *Arabidopsis*, and a regulatory network formed from different flower pathways has been described (Boss et al. 2004; Jack 2004; Moon et al. 2005; Putterill et al. 2004). To identify flowering-related genes in litchi, we used unigene sequences in BLAST searches of the National Center for Biotechnology Information Nr protein datasets and found that at least 188 unigenes had sequence similarities to known flowering-related genes from other plants (Table S9). Most of the flowering- related genes identified in the present study were assigned to four known flowering-related pathways, and the representation of the relationships among these pathways and the putative homologs of the key flowering-related genes in litchi are presented in Fig. 7; Table S9.

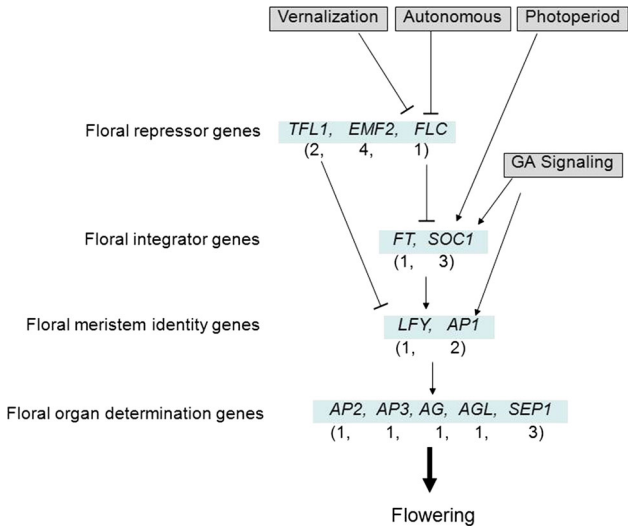

Fig. 7 A simplified diagram showing the four major genetic pathways regulating flowering time in *Arabidopsis* (Putterill et al. 2004; Boss et al. 2004). *Arrows* indicate that activation and *straight lines ending with a perpendicular line* indicate repression. All expressed genes involved in these flowering pathways are listed in Table S6. The *numbers below each gene* represent the members of the corresponding genes found from RNA-seq data for litchi

For the photoperiod pathway, many unigenes were found to have similarities to photoreceptor and circadian clock components, including *PHYTOCHROME A* (*PHYA*), *LATE ELONGATED HYPOCOTYL* (*LHY*), *PSEUDO-RESPONSE REGULATOR 1* (*PRR95*), *EARLY FLOWERING 3* (*ELF3*), *EARLY FLOWERING 4* (*ELF4*), *EARLY FLOWERING 6* (*ELF6*), *FLAVIN-BINDING KELCH REPEAT F-BOX 1* (*FKF1*), *TIMING OF CAB 1* (*TOC1*), and *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*COP1*). No sequences similar to *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *GIGANTEA* (*GI*) were identified. Furthermore, several litchi sequences similar to *CONSTANS* (*CO*), the key regulator of the photoperiod response and the common upstream regulators of *FT* and *SOC1*, were also found (Table S9).

A putative homolog of *FLC* in the vernalization pathway was identified in this study. *FLC*, a flowering repressor integrating the autonomous flowering and vernalization pathways, severely delays flowering by repressing the expression of *FT* and *SOC1*. In addition, several litchi sequences exhibited similarities to genes in the vernalization pathway, including *VERNALIZATION-INDEPENDENT INSENSITIVE 3* (*VIN3*), *VERNALIZATION 1* (*VRN1*), *EMBRYONIC FLOWER 2* (*EMF2*), *FERTILIZATION INDEPENDENT ENDOSPERM* (*FIE*), and *MULTICOPY SUPPRESSOR OF IRA 1*(*MSI1*), which are involved in regulating *FLC* expression. Putative autonomous flowering pathway genes were identified, including *LUMINIDEPENDENS-like* (*LD*), *FY*, *FCA*, *FPA*, and *DICER-LIKE 1* (*DCL1*). Moreover, putative homologs of GA pathway genes were identified, including the GA response modulators *GA3 oxidase* (*GA3ox*), *SPINDLY* (*SPY*), and GA receptor DELLA protein *GAI1-like* (*GAI*) (Table S9).

In addition to finding genes related to the four known flowering pathways, we considered the identification of candidates for floral integrator genes such as *SOC1* and *FT*, and floral meristem identity genes such as *LFY* and *AP1* particularly important (Fig. 7; Table S9). *LFY* and *AP1* are the two major floral meristem regulators in *Arabidopsis*, and overexpression of *LFY* and *AP1* causes early formation of floral meristems (Mandel and Yanofsky 1995; Weigel 1995). Two orthologous genes of *TERMINAL FLOWER 1* (*TFL1*), a floral repressor in *Arabidopsis*, were also found in our transcriptome data. Some additional non-classified flowering regulators including members of the MADS transcription factor, *AP2-EREBP* transcription factor and *SQUAMOSA PROMOTER-BINDING PROTEIN* were also identified (Table S9). These families represent a large group of plant development regulators, particularly genes that determine floral organ development (Becker and Theissen 2003; Honma and Goto 2001; Melzer et al. 2010; Pelaz et al. 2000; Rounsley et al. 1995). Most flowering-related unigenes exhibited slight expression differences between the two phases (Table S9). To verify the results of differential gene expression analysis obtained from RNA-Seq data, we selected nine genes known to have important roles during floral transition for q-PCR analysis (Fig. 8). In phase 2, the expression of most flowering-promoting genes was obviously up-regulated compared with phase 1. Particularly, floral meristem identity genes (*LFY* and *AP1*) were clearly up-regulated in the phase 2. On the other hand, the repressors of flowering genes (*FLC*, *EMF* and *TFL1*) were slightly down-regulated during floral initiation (Fig. 8). However, some results from the expression patterns determined by q-PCR were not strictly consistent with those obtained by RNA-Seq analyses (Fig. 8). To explain the significant difference, we selected twenty-one differentially expressed genes related to floral transition to further confirm the accuracy of the transcriptomic data. The correlation between transcriptome analysis and q-PCR was evaluated, by comparing the $\log_2$ fold change between transcriptome results and q-PCR. There was a positive correlation between the two methods (Fig. S6), suggesting changes in most genes are consistent between the two methods.

Discussion

Little genomic information is available for litchi and even the network regulating floral initiation in this species is missing. An increasing number of studies have shown that Illumina sequencing is a powerful tool for global analysis

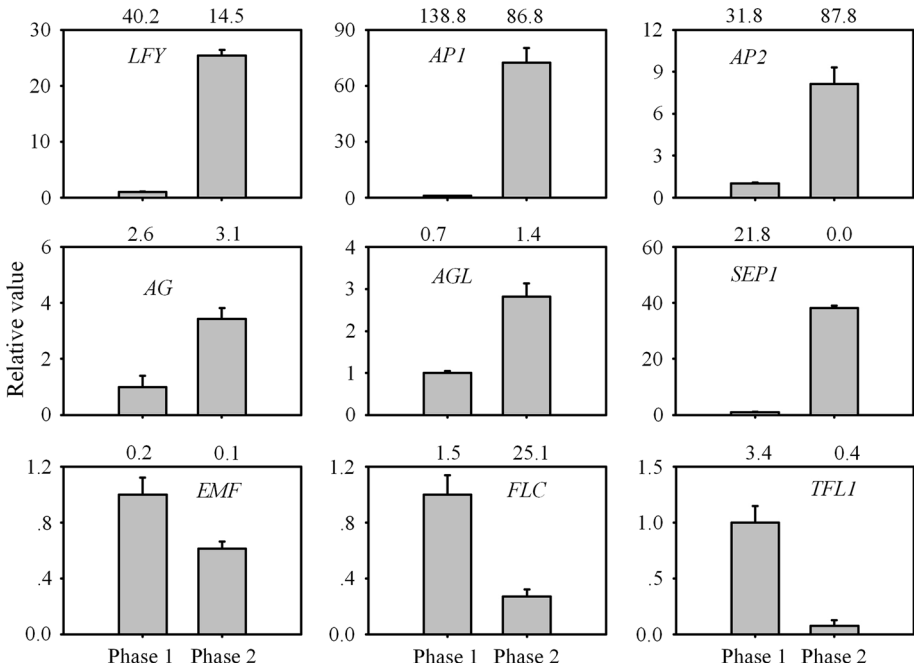

Fig. 8 Real-time quantitative expression of selected flower-related genes. The RPKM value of two samples from RNA-Seq data is listed above each gene. Relative transcript levels are calculated by q-PCR using β-actin as a standard. *Error bars* indicate the standard deviation of three technical replicates

of molecular mechanisms during different development stages (Wong et al. 2013; Zhang et al. 2012).

This study used RNA-Seq technology to profile the litchi buds transcriptome on the Illumina HiSeq^{TM} 2000 platform, and obtained approximately 87 million reads. 41,347 out of 53,453 unigenes were successfully annotated against public databases, suggesting their relatively conserved functions. 5,928 differentially expressed genes were identified between the two bud phases, including 4,622 unigenes as floral activators and 1,306 unigenes as floral repressors. This is the first attempt using Illumina sequencing technology to gain insight into the transcriptome of litchi flower buds, although the transcriptome of litchi fruit has provided a global description of fruit development (Li et al. 2013a, b). KEGG pathway enrichment analysis indicated that pathways related to the biosynthesis of five phytohormones were significantly enriched. Generally, the levels of several unigenes annotated as genes that are related to hormone biosynthesis, transport, or signaling changed during floral transition. Auxin plays a pivotal role during plant flower morphogenesis, as it defines the site of flower initiation (Alabadi et al. 2009; Okada et al. 1991; Vanneste and Friml 2009). Genes involved in auxin transport or signaling were up-regulated in the shoot apical meristem during floral initiation in rose (Dubois et al. 2011) and soybean (Wong et al. 2009). In agreement with these findings, our study also showed increased transcript abundance of IAA biosynthesis genes and IAA responsive genes, including putative *tryptophan synthase*, *indole-3-acetic acid* (*IAA*)-*amino acid hydrolase*, and *YUCCA*, during floral initiation (Table 2). *YUCCA* encodes the enzymes catalyzing the rate-limiting step in the auxin biosynthesis pathway (Cheng et al. 2006). An increase in auxin level could subsequently trigger the upregulation of auxin-responsive transcripts including putative *ARFs*, *Aux/IAA*, *GH3*, and *SAUR* (Table 2) (Fig. 5c). The gradual increases in *ARF* and *Aux/IAA* transcript abundance during floral initiation were then confirmed by q-PCR (Fig. 6). These results suggested that an increase in auxin levels may occur during floral initiation, and auxin may play an important role in the shoot apex during floral initiation in litchi, as be verified in soybean (Wong et al. 2013).

CK has been found to promote flowering in *Arabidopsis* (Bartrina et al. 2011; Corbesier et al. 2003). Endogenous cytokinin levels increased in buds during floral initiation, and exogenous applications facilitated floral initiation in litchi (Chen 1991). In our results, there was an overall increase in transcripts of genes related to cytokinin biosynthesis suggesting an increase in CK concentrations in the shoot apical meristem (SAM) during floral initiation (Table 2). Transcripts of genes annotated as *triosephosphate isomerase*, *Cytokinin oxidase,* and *gulonolactone oxidase* were significantly up-regulated in phase 2. Of the unigenes related to CK signaling, *ARR* and *HK4* were also found to be up-regulated during floral initiation (Fig. 5e). These alterations suggested the involvement of cytokinin in litchi floral initiation. However, no significant upregulation of CK biosynthesis genes was observed in the soybean apical meristem during floral initiation (Wong et al. 2013).

The role of ABA in flowering is ambiguous. An increase in ABA level and upregulation of ABA biosynthesis and

signaling were reported in the SAM during the floral initiation process in soybean (Wong et al. 2009, 2013). *ABA-insensitive8* (*abi8*) mutants of *Arabidopsis* with defects in a positive regulator of ABA signaling displayed delayed flowering (Brocard-Gifford et al. 2004). However, a repressive role of ABA in flowering had been presented by Razem et al. (2006), and the interaction of flowering-time control protein A (FCA) with ABA resulted in a delay of the flowering process in *Arabidopsis*. FCA is a nuclear RNA-binding protein involved in flowering, and as an ABA receptor binds ABA with high affinity (Razem et al. 2006). Our study showed decreased transcript abundance of ABA biosynthesis genes (*NCED* and *CCD*) and increased abundance of ABA catalysis gene (*CYP707A*) transcripts during floral initiation (Table 2). These alterations, together with the changes in the expression of ABA signaling genes, suggested that a relatively low ABA level corresponds to floral initiation.

In addition, we found that a range of SA-related and JA-related genes isolated in our study such as *PAL*, *linoleate 13S-lipoxygenase*, *jasmonate-O-methyltransferase*, *JAZ*, *MYC2*, *PR-1*, *NPR1* and *TGA* were differentially regulated during floral initiation in litchi (Table 2; Fig. 5). A high accumulation of JA in the apical meristem during floral initiation has been reported in soybean (Wong et al. 2009). Previous studies also indicated that JA may play a role in regulating the floral initiation process (Krajncic et al. 2006). Taken together, these findings suggest that floral initiation in litchi is subjected to complex regulation by multiple hormones. The regulatory mechanisms of phytohormones in litchi floral initiation process are still far from elucidated. The identification of genes of plant hormone signaling pathways will facilitate future studies of floral initiation in litchi. The challenges will be to identify the way in which these hormones signaling events integrate into the current floral regulatory pathways.

In this study, we detected 188 flowering-related gene homologs, based on sequence annotations and analyses of changes in gene expression during floral initiation of litchi. Some of these genes encode regulators involved in four flowering-time pathways and some are putative homologs for floral integrator or identity gene, while others are related to flower development.

In *Arabidopsis*, the floral induction signals arise mainly from four major flowering pathways (photoperiod, autonomous, vernalization, and GA-induced pathways). These flowering-related genes were identified in our study, suggesting that all the four flowering pathways may be present in litchi as well. Several key floral candidate genes such as *SOC1*, *FT*, *LFY* and *AP1* also were found (Fig. 7; Table S9). FT protein is mainly expressed in the vascular tissues of the leaf, and is then transported via the phloem to the shoot apical meristem where it triggers flower development (Corbesier et al. 2007). Interestingly, we detected *LcFT1* gene expression in litchi bud tissue, and quantitative analysis showed that the expression level of *LcFT1* in bud tissue was far lower than that in the corresponding leaves during the whole process of flower formation (unpublished data). Similarly, Zhang et al. (2008) examined an *FT* homologous gene in orange by in situ hybridization, and the results showed little *FT* expression in the meristems of the vegetative growth phases, but the low expression of *FT* was restricted to the apical meristems in the floral determination phase. Expression of *FT* homologs of Pea was also detected in shoot apices (Hecht et al. 2011). However, the existence and role of *LcFT1* in litchi flower buds require further study.

LFY and *AP1* are two major floral meristem regulators, and their overexpression caused early flowering in transgenic *Arabidopsis* (Benlloch et al. 2007). *AP1*, mainly activated by *FT*, is also necessary for establishing and maintaining flower meristem identity (Mandel and Yanofsky 1995; Weigel 1995). Expression analysis of *LFY* and *AP1* by q-PCR showed that the transcript levels of the two genes were significantly up-regulated during the floral initiation (Fig. 8). These results suggested the relatively conserved functions of these key regulators in the floral meristem of both *Arabidopsis* and litchi.

We also found some litchi homologs for flowering development genes in this study, and the results of q-PCR showed that the transcript levels of *AP2*, *AG*, *AGL*, *SEP1* were significantly up-regulated in the flower buds (Fig. 8), as shown in soybean (Wong et al. 2013), whereas these genes mainly functioned in flower morphogenesis in *Arabidopsis* (Becker and Theissen 2003; Honma and Goto 2001; Melzer et al. 2010; Pelaz et al. 2000). The fact that upregulation of these transcripts occurred earlier than expected in the litchi floral initiation process might indicate a different floral developmental plan in litchi. The floral repressors, including *FLC, EMBRYONIC FLOWER2* (*EMF2*), and *TERMINAL FLOWER 1* (*TFL1*), were expressed in the floral initiation phase in litchi (Table S9). In Arabidopsis, overexpression of *FLC* resulted in extremely delayed flowering by repressing the expression of the floral activators *FT* and *SOC1* (Amasino 2010; Helliwell et al. 2006). *EMF* may act to delay flowering by interacting with floral meristem identity genes (Sung et al. 1992; Yang et al. 1995). *TFL1* plays a major role in counteracting the establishment of floral meristem identity and delays flowering time by repressing *LFY* and *AP1* (Liljegren et al. 1999; Shannon and Meeks-Wagner 1991). From the results of q-PCR, it was clear that the transcript levels of the repressors are slightly down-regulated during floral initiation (Fig. 8). In addition, epigenetic overexpression of the *FLC* gene of litchi was found to strongly delay flowering in *Arabidopsis* (unpublished data). The results indicated that

these floral repressors may play critical roles in flower initiation in litchi.

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Conflict of interest The authors declare that they have no conflict of interest.

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