Ornamental
Plant Research

ARTICLE

https://doi.org/10.48130/OPR-2021-0007
Ornamental Plant Research **2021**, 1: 7

Identification and functional verification of *PlFT* gene associated with flowering in herbaceous peony based on transcriptome analysis

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Abstract

The herbaceous peony (*Paeonia lactiflora* Pall.) is considered to be a highly valued cut flower plant. It has large flower with rich colors. However, there has been little or no research into the genes related to its flower development. In this study, we used the Illumina HiSeq platform to analyze the RNA-Seq comparative transcriptome of the *P. lactiflora* 'Dafugui' in three different flowering periods. Nine cDNA libraries were established, from which 92.53 Gb data with 81,788 unigenes were obtained. We screened the genes related to *P. lactiflora* flowering, isolated and cloned the *PlFT* gene related to flowering. The total length of the *PlFT* gene was 592 bp, which had a complete open reading frame of 522 bp and encoded 173 amino acids. The accession number of the *PlFT* gene is MT249229. To test the role of *PlFT*, we constructed an expression vector for genetic transformation. Its expression in Arabidposis mutant indicated that *PlFT* was involved in the flowering of *P. lactiflora*. This is the first transcriptome analysis of flower development in *P. lactiflora*. Our results provide some fundamental information for further analyzing the molecular mechanism underlying flower development of *P. lactiflora*.

Citation: Sun J, Chen T, Wu Y, Tao J. 2021. Identification and functional verification of *PlFT* gene associated with flowering in herbaceous peony based on transcriptome analysis. *Ornamental Plant Research* 1: 7 https://doi.org/10.48130/OPR-2021-0007

INTRODUCTION

The herbaceous peony (*Paeonia lactiflora* Pall.), a member of the family Paeoniaceae, is a traditional herb flowering plant and has a long history of cultivation in China. It used to grow in the Imperial Palace Garden and is known as the 'Prime-Minister of flowers'[1]. The *P. lactiflora* flower is elegant and beautiful with a high ornamental value. Its flowers have not only single petal, double petal, golden pistil, crown, and other flower types but also rich colors, such as white, green, pink, and yellow. Regulation of flowering of herbaceous peony can be manipulated in protected cultivation, but the production cost is high. *P. lactiflora* loses the stems and leaves in autumn but survives in winter as a dormant root mass as the herbaceous peony is deciduous[2]. To break the dormancy of buds in spring, it needs a long time of low temperature during dormancy to meet the winter-cold requirements[3–5]. In addition to the low temperature, application of GA_3 can promote flowering[6]. For the early flowering cultivar Dafugui, 4–5 axillary buds on the top of the terminal bud developed into a primordium shape in the overwintering state. 'Dafugui' experiences flower bud differentiation, bract primordium differentiation, petal primordium formation, stamen primordium emergence and development, and pistil primordium formation from early September to April of the following year[7]. Information on flower bud differentiation and flowering regulation of herbaceous peony is helpful to provide the theoretical basis for promoting flowering.

Flowering marks the transition from vegetative growth to reproductive growth, and the time of flowering affects a plants commercial value[8]. At the same time, regulation of the flowering period is also essential for plant seed setting. Compared to other species, the molecular mechanism of flowering in *Arabidopsis* is relatively well understood[9]. In *Arabidopsis*, there are six response pathways including photoperiod, vernalization, autonomic, temperature, gibberellin, and age pathway, to regulate flowering[10]. Both endogenous and exogenous factors determine the flowering time of plants. Endogenous factors are mainly related to the genes that regulate flowering transformation. For example, *FT*[11], *FLC*[12], *LFY*[13] and *SOC1*[14] are called flower induction switches. Among them, FT protein is a globular protein with 175 amino acids, both mRNA and FT protein can regulate flowering. *FT* gene is regarded as a florigen gene[15], which is conservative. It was previously reported that the FT protein could be transported between cells through phloem and transported to the apical meristem, inducing plant flowering[16]. It does not work alone, but interacts with other proteins to regulate flowering.

Currently, *FT* and its homologous genes are found to promote flowering in winter wheat, barley[17], rubber tree[18], rice[19,20], vanda hybrid[21], chrysanthemums[22] and other plants. It is reported that the *LFT1* gene of *Lilium* was most homologous to the *AtFT* gene and showed peak expression in shoot apices, which promotes flowering in *Arabidopsis*[23]. The regulation of flowering by *FT* is also influenced by photoperiod induction and other proteins. Recently, Jing et

al. found that *PKL* could interact with *CO* and bind to *FT* to mediate *FT* response to photoperiod induced flowering and counteract the inhibitory activity of PcG protein, resulting in appropriate *FT* expression and flowering response[24]. *FT* has some changes in species evolution, so not all *FT* homologous genes have or only have a promotive effect. A study on onion has shown that the up-regulation of *AcFT2* can induce vernalization and promote flowering, while *AcFT1* and *AcFT4* are related to bulb formation[25]. In addition, *GmFT1a* was found to inhibit flowering in soybean, which was a flower inhibitory factor[26].

Transcriptome sequencing is a high-throughput technology for analyzing gene sequence data and can be divided into three categories: synthetic sequencing, solid-state sequencing, and single-molecule sequencing[27]. Illumina platform is the most widely used platform based on data quality, quantity and cost[28]. As an efficient and high-throughput method for functional gene mining and pathway analysis, transcriptome sequencing was introduced into many studies of horticultural research. There were many types of research on mining flowering genes through transcriptome in some species including sweet potato[29], bamboo[30,31], *Lagerstroemia indica*[32], *Ipomoea nil*[33] and *Eichhornia paniculate*[34]. For example, in Singh & Jain's previous research, the RNA-seq of various stages of flower development and few vegetative tissues in chickpea found differentially expressed genes related to various biological processes and molecular functions during flowering[35]. Zhang et al. reported that 110 citrus flowering-time genes homologous with known elements of flowering-time pathways were identified by sequencing and bioinformatics analysis in *Poncirus trifoliata* (L.) Raf[36]. High throughput transcriptome sequencing plays an important role in the study of flowering regulation.

In this study, transcriptome sequencing technology was used to analyze different gene expression during the flowering of *P. lactiflora* and to identify those involved in regulation of flower development. An important gene, *PlFT* was isolated and cloned for genetic transformation in Arabidopsis. Its expression in relation to flower development in *Arabidopsis* indicated that *PlFT* could play an important role in regulating flowering in *P. lactiflora*.

RESULTS

Sequencing read filtering and *de novo* assembly

Nine cDNA libraries (three biological repeats) were constructed by collecting samples from T1, T2 and T3 to elucidate *P. lactiflora* 'Dafugui' flowering mechanism (PRJNA723469). T1 is the critical period of flower bud differentiation, T2 is the period of flower bud morphogenesis, and T3 is the period of flower opening (Fig. 1a). A total of

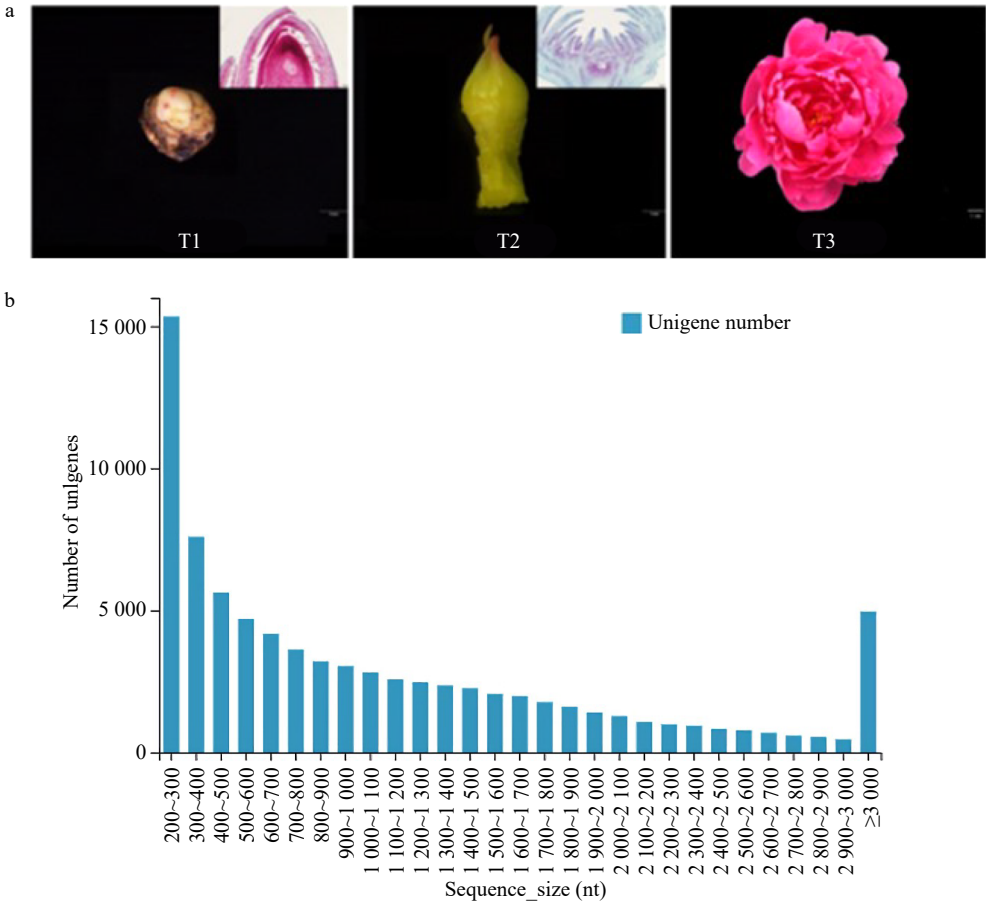

Fig. 1 Transcriptome sequencing of 'Dafugui'. (a) Materials of 'Dafugui' in three periods. T1, critical period of flower bud differentiation; T2, flower bud morphogenesis; T3, peak flowering. (b) Unigene length distribution.

92.53 GB of data were obtained by the Illumina Hiseq platform. After assembling the original data and removing the redundancy of low quality, joint pollution, and high content of unknown base N in the original data, the filtered reads quality statistics were obtained, as shown in Supplemental Table 1. After filtering, the percentage of reads was > 92.6%, and the percentage of Q20 (%) > 97.9%. Overall, the percentage of bases with low quality was lower, indicating that the sequencing quality was sound. Since *P. lactiflora* has no reference genome, it was necessary to splice the clean reads to obtain the reference sequences for subsequent analysis; the results are shown in Supplemental Table 2. A total of 81,788 unigenes containing 93,770,651 nt were obtained, with an average length of 1,146 nt. N50, N70, and N90 were 1,780 bp, 1,193 bp and 520 bp, respectively. Fig. 1b shows the length distribution statistics of unigenes. The sequence length of unigenes ranged from 200 nt to 3,000 nt. With the increase of sequence size, the number of unigenes gradually decreased, but there was no apparent separation. All the above results indicated that RNA sequencing has good continuity and high quality.

Gene functional annotation

The assembled unigenes were compared to seven function databases (KEGG, GO, NR, NT, SwissProt, Pfam and KOG) for annotation to understand the function information. Finally, 52,323 (NR: 63.97%), 35,949 (NT: 43.95%), 39,639 (SwissProt: 48.47%), 43,118 (KOG: 52.72%), 41952 (KEGG: 51.29%), 40,116 (GO: 49.05%) and 40,206 (Pfam: 49.16%) unigenes were annotated. Fig. 2a shows the Venn diagram of the number of unigenes annotated by KEGG, GO, NR, Swissprot, and KOG.

The corresponding functional annotation was obtained by comparing unigenes sequences with the NR database. According to the results of NR functional annotation, the proportion of different species in unigenes annotation was counted, and the species distribution map was drawn (Fig. 2b). The unigenes of *P. lactiflora* 'Dafugui' were similar to those of five plants, *Vitis vinifera* (21.41%), *Quercus suber* (8.89%), *Actinidia* (4.09%), *Juglans regia* (3.91%) and *Nelumbo nucifera* (2.79%).

Unigenes were annotated into the KOG database and were classified into 25 functional processes (Fig. 2c). Among them, 9,048 unigenes were compared to the 'general function prediction only', followed by 4,598 unigenes to the variety of 'signal transmission mechanisms', and 4,048 unigenes to the category of 'posttranslational modification, protein turnover, and chaperones'.

Analysis of differentially expressed genes for the development of herbaceous peony 'Dafugui' flowers

The FPKM values of unigenes from different stages (T1, T2, and T3) were compared. The DEGs were screened to obtain the flowering-related genes of *P. lactiflora*. The differences between the T1, T2 and T3 groups are shown in Supplemental Fig. 1. The number of differentially expressed genes is shown in Supplemental Table 3. Compared with T1 and T2, there

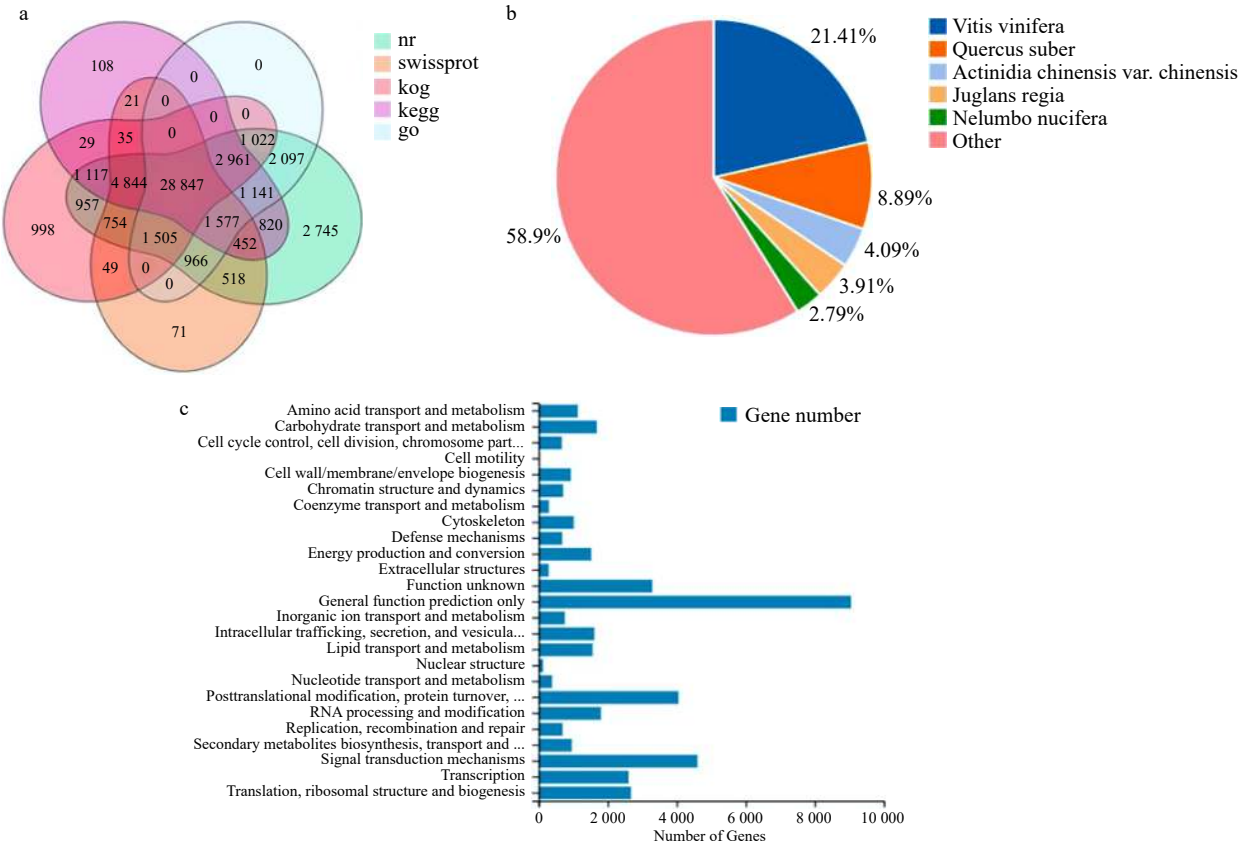

Fig. 2 Gene functional annotation. (a) Venn diagram of the number of unigenes annotated in different public databases. (b) Species distribution of NR annotation. (c) Functional classification of KOG annotation. The x-axis represents the corresponding number of Unigene, the y-axis represents the KOG function classification name.

were 28,075 DEGs, including 19,821 up-regulated and 8,254 down-regulated. Compared with T1 and T3, there were 40,666 DEGs, including 22,171 up-regulated and 18,495 down-regulated. There were 37,230 DEGs between T2 and T3, of which 15,064 up-regulated and 22,166 down-regulated.

GO and KEGG Pathway analysis of DEGs

All DEGs were annotated by GO and were classified into three functional categories: molecular function, cellular component, and biological process (Fig. 3a). Moreover, all DEGs were also annotated by the KEGG pathway. The top 20 pathways are shown in Fig. 3b. During the three periods, five metabolic pathways were enriched, including 'circadian rhythm-plant', 'carotenoid biosynthesis', 'flavonoid biosynthesis', 'isoflavonoid biosynthesis' and 'MAPK signaling pathway'.

To explore the key genes of flowering regulation during flower bud differentiation of *P. lactiflora* 'Dafugui' and the molecular mechanism of flowering regulation, the DEGs in T1 vs T2, T1 vs T3, and T2 vs T3 were analyzed, and the up-down relationship was marked (Table 1). Twenty three DEGs were screened and annotated by KEGG, including *CHE*, *PHYB*, *LHY*, *CO*, *PRR5*, *PRR7*, *FKF1*, *CDF1*, *PHYA*, *PAP1*, *HY5*, *TOC1*, *ELF3*, *GI*, *FT*, *CRY1*, *CRY2*, *ZTL*, *SPA1*, *FLC*, *FUL*, *AP1* and *SOC1*, which were related to flower development.

Verification of transcriptome reliability by qRT-PCR

qRT-PCR was used to verify the reliability of transcriptome sequencing. Nine DEGs related to flowering were randomly selected, and the samples of T1, T2 and T3 were used to analyze their expression. The nine DEGs were *TCP21*, *CHE*, *CO*, *PRR5*, *PAP1*, *MYB75*, *HY5*, *ELF3*, *GI*, *FT* and *CRY1*. As shown in Fig. 4, the expression changes of nine random genes of three periods were highly consistent with the transcriptome sequencing results, indicating that the transcriptome sequencing results obtained were accurate and reliable.

Sequence analysis of one flowering-related gene in *Paeonia lactiflora*

Florigen (*FT*), known as a flowering hormone, plays a crucial role in the flowering gene network. FT protein is synthesized in plant leaves, transported over a long distance, and accumulated at the shoot apical meristerm (SAM) to trigger the process of flower bud differentiation. In our transcriptomic data, *PlFT* gene expression was significantly different in flower developmental stages, which might indicate that it could play an important role in the formation of peony flowers. Therefore, we isolated *PlFT* and carried out further functional research. The total length of the *PlFT* gene was 592 bp, whose complete open reading frame was 522 bp and encoded 173 amino acids. The amino acid sequences of *PlFT* were compared with those of *Paeonia suffruticosa*, *Rosa chinensis*, *Malus domestica,* and *Petunia x hybrida* by DNAMAN software (Fig. 5a). The amino acid sequences of *PlFT* were 99%, 93%, 93%, and 89% similar to those of *PsFT* (AHM25242.1), *RcFT* (XP_024189593.1), *MdFT* (NP_001280810.1) and *PhFT* (AZL87173.1), respectively. The similarity between *FT* and

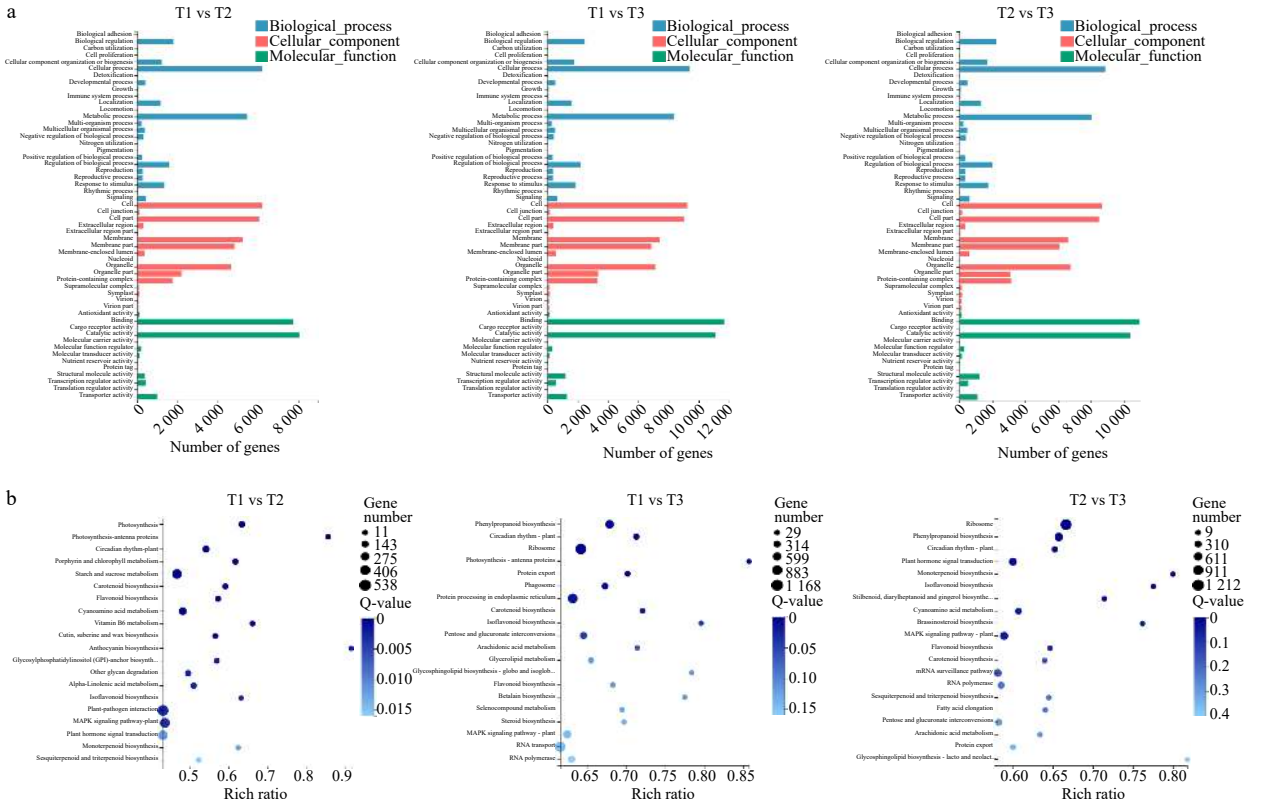

Fig. 3 GO and KEGG Pathway analysis of DEGs. (a) Functional distribution of DEGs annotated GO. The x-axis represents the number of genes annotated GO and the y-axis represents the functional distribution of GO. (b) Bubble diagram of enrichment of DEGs KEGG pathway. The x-axis is enrichment ratio, the y-axis is KEGG Pathway, the bubble size indicates the number of genes annotated on one KEGG pathway, the color represents enrichment Q-value, and darker color represents smaller Q-value.

Table 1. Analysis of main DEGs for KEGG pathway related with flowering of 'Dafugui' samples in different periods.

Gene ID	Entry	Name	log_2(T2/T1)	log_2(T3/T1)	log_2(T3/T2)	T1 vs T2	T1 vs T3	T2 vs T3
CL1090.Contig1_All	K16221	*TCP21, CHE*		−1.22	−1.88		down	down
CL1090.Contig2_All				−1.70	−2.21		down	down
Unigene12923_All				−3.09	−2.39		down	down
Unigene15030_All			2.53	2.33		up	up	
Unigene17026_All					−1.13			down
Unigene19482_All				−3.00	−3.60		down	down
Unigene6346_All				2.27	1.44		up	up
CL1148.Contig1_All	K12121	*PHYB*	4.43	11.10	6.68	up	up	up
CL1148.Contig4_All			1.58	7.06	5.48	up	up	up
CL1516.Contig10_All			2.70	7.40	4.70	up	up	up
CL1516.Contig11_All			4.12	8.90	4.78	up	up	up
CL1516.Contig2_All			2.97	1.25	−1.72	up	up	down
CL1516.Contig3_All			5.19	9.32	4.12	up	up	up
CL1516.Contig4_All				6.56	6.69		up	up
CL1516.Contig5_All			−2.89	−6.12		down	down	
CL1516.Contig6_All			−8.14	−8.16		down	down	
CL1516.Contig7_All			−3.44	5.73	9.17	down	up	up
CL1516.Contig8_All			1.11	−1.54	−2.65	up	down	down
CL1516.Contig9_All			1.84	5.98	4.13	up	up	up
CL175.Contig2_All			2.40	2.36		up	up	
CL175.Contig4_All				2.26	1.91		up	up
CL5438.Contig1_All			−1.79	−8.85	−7.07	down	down	down
CL6053.Contig1_All				1.19			up	
CL6053.Contig2_All			2.58	4.17	1.59	up	up	up
CL6053.Contig3_All			−1.34	1.94	3.28	down	up	up
CL6068.Contig1_All				4.60	4.51		up	up
CL6900.Contig1_All			1.30	−7.36	−8.65	up	down	down
CL6900.Contig2_All			1.09	1.36		up	up	
CL7074.Contig1_All				8.14	8.04		up	up
CL7690.Contig3_All				−1.93	−2.80		down	down
CL7690.Contig4_All			1.01	−7.93	−8.94	up	down	down
CL7690.Contig5_All				1.54			up	
Unigene12459_All			1.26		−1.04	up		down
Unigene12460_All				6.55	6.45		up	up
Unigene12461_All			3.70	8.02	4.32	up	up	up
Unigene1351_All				9.45	9.35		up	up
Unigene15547_All				9.58	9.48		up	up
Unigene24958_All			−4.59	2.60	7.20	down	up	up
Unigene25706_All				8.96	7.86		up	up
Unigene39640_All					−1.07			down
Unigene6288_All			1.24			up		
Unigene6289_All			1.29			up		
CL1150.Contig3_All	K12133	*LHY*	3.80	3.14		up	up	
CL1150.Contig4_All			3.98	4.05		up	up	
CL1150.Contig5_All			6.15	6.25		up	up	
CL1150.Contig7_All			4.35	4.23		up	up	
CL1184.Contig1_All			2.71		−2.01	up		down
CL5076.Contig1_All					−1.34			down
CL5076.Contig3_All					−1.62			down
CL5076.Contig4_All			−2.16	−7.47	−5.31	down	down	down
CL5076.Contig5_All			−1.21			down		
CL8141.Contig1_All			−1.09	2.51	3.60	down	up	up
CL8141.Contig2_All				2.33	2.79		up	up
CL1267.Contig1_All	K12135	*CO*	1.16	2.88	1.72	up	up	up
CL1672.Contig2_All			1.13			up		
CL3591.Contig1_All			1.59	−1.09	−2.68	up	down	down
CL3591.Contig2_All			2.67	−2.06	−4.73	up	down	down
CL3591.Contig3_All			2.35	−6.78	−9.13	up	down	down
CL3591.Contig4_All			3.10	−1.09	−4.19	up	down	down
CL3591.Contig5_All				−1.60	−2.47		down	down
CL409.Contig3_All				−1.11	−1.56		down	down
CL409.Contig4_All					−1.39			down

(to be continued)

Table 1. (continued)

Gene ID	Entry	Name	log_2(T2/T1)	log_2(T3/T1)	log_2(T3/T2)	T1 vs T2	T1 vs T3	T2 vs T3
CL409.Contig5_All			1.21		−1.52	up		down
CL4357.Contig1_All			7.30	8.69	1.38	up	up	up
CL4357.Contig2_All			4.39	3.23	−1.16	up	up	down
CL4357.Contig3_All			9.23	11.51	2.28	up	up	up
CL4357.Contig4_All			2.96	4.33	1.37	up	up	up
CL4357.Contig5_All			5.08	1.55	−3.52	up	up	down
CL6692.Contig3_All			2.25	−4.50	−6.75	up	down	down
CL7718.Contig1_All			1.65	−1.49	−3.14	up	down	down
CL7718.Contig2_All			1.44		−1.29	up		down
CL7917.Contig1_All				−1.38			down	
CL7917.Contig2_All				−1.28	−1.01		down	down
CL7917.Contig3_All				−1.75	−1.79		down	down
CL7932.Contig1_All			1.92	2.14		up	up	
CL7932.Contig2_All			1.95	2.86		up	up	
Unigene11310_All			2.14		−2.24	up		down
Unigene1286_All			1.84	1.25		up	up	
Unigene14242_All			3.42	7.71	4.29	up	up	up
Unigene17669_All			6.48	4.58	−1.90	up	up	down
Unigene32926_All			4.14		−5.07	up		down
Unigene410_All			5.11	5.13		up	up	
Unigene496_All			2.47		−2.83	up		down
Unigene9300_All			1.86	7.00	5.14	up	up	up
CL3891.Contig1_All	K12130	*PRR5*	7.17	4.47	−2.70	up	up	down
CL3891.Contig2_All			3.36		−7.07	up		down
CL3891.Contig3_All			2.69		−2.64	up		down
CL3891.Contig4_All			2.56		−2.46	up		down
CL6193.Contig1_All			4.75	3.70	−1.05	up	up	down
CL6193.Contig2_All			4.31	2.52	−1.78	up	up	down
Unigene8183_All			2.55		−2.91	up		down
Unigene8184_All			3.33		−2.79	up		down
CL4229.Contig1_All	K12129	*PRR7*	1.21	2.86	1.65	up	up	up
CL4229.Contig10_All			1.44	3.38	1.94	up	up	up
CL4229.Contig2_All			1.59	3.96	2.38	up	up	up
CL4229.Contig3_All			1.46	2.59	1.13	up	up	up
CL4229.Contig4_All			1.83	1.78		up	up	
CL4229.Contig5_All			4.09	4.59		up	up	
CL4229.Contig6_All			3.02	−6.05	−9.07	up	down	down
CL4229.Contig7_All			3.20	6.69	3.48	up	up	up
CL4229.Contig8_All			2.51	2.28		up	up	
CL4229.Contig9_All			6.17	7.13		up	up	
CL7872.Contig1_All			1.32	2.56	1.24	up	up	up
CL7872.Contig2_All			2.34	3.67	1.33	up	up	up
Unigene6980_All			4.16	2.26	−1.90	up	up	down
CL4436.Contig1_All	K12116	*FKF1*		−1.34	−1.58		down	down
CL4436.Contig2_All			1.13		−1.20	up		down
CL4719.Contig1_All	K16222	*CDF1*	−1.27		1.27	down		up
CL5753.Contig1_All				3.95	2.85	down	down	
CL5753.Contig2_All				−2.77	−3.64	down	down	down
CL5837.Contig1_All				−1.83	−2.67	up	up	up
CL5837.Contig2_All			−3.49	−5.83		up	up	up
CL6631.Contig2_All				−3.77	−3.58		down	down
CL8648.Contig1_All				−1.90		down	down	down
CL8648.Contig2_All					−1.36	down	down	down
Unigene10801_All			10.91		−7.84		up	up
Unigene11044_All			−2.71	−8.05	−5.34		up	
Unigene12006_All				7.60	5.50	up		down
Unigene12046_All			−2.30	−1.32		up	up	up
Unigene12573_All			−2.04	−4.46	−2.43	down	down	up
Unigene14940_All			7.05	10.78	3.73	up		down
Unigene14950_All			7.24	13.54	6.30	down	down	down
Unigene695_All				−2.86	−2.83	up	up	

(to be continued)

Table 1. (continued)

Gene ID	Entry	Name	log_2(T2/T1)	log_2(T3/T1)	log_2(T3/T2)	T1 vs T2	T1 vs T3	T2 vs T3
Unigene972_All			−2.18	−6.64	−4.47	up	up	
Unigene15046_All			−2.45	−5.86	−3.41	up		down
CL5158.Contig1_All	K12120	*PHYA*		1.44	1.08		up	up
CL9769.Contig1_All				1.65			down	down
CL9769.Contig2_All			1.95		−1.86		down	down
CL9769.Contig3_All			4.27	5.83	1.56	down	down	
Unigene14810_All			−3.09	−1.66	1.43		down	down
Unigene2317_All			2.03		−2.02		down	
Unigene2317_All			−3.39	−5.28	−1.89			down
CL5739.Contig1_All	K16166	*PAP1, MYB75*	1.94	2.48		up		down
CL5739.Contig3_All			1.89	1.64		down	down	down
Unigene15771_All			1.81		−1.74		up	up
CL6391.Contig1_All	K16241	*HY5*	3.25	2.69		up	up	
CL6391.Contig2_All			4.25	1.38	−2.87	up	up	down
CL6391.Contig3_All			5.39		−4.60	up		down
CL9659.Contig1_All			−1.22	−1.90		down	down	
Unigene6996_All			8.00	7.61		up	up	
CL6767.Contig1_All	K12127	*TOC1, APRR1*	2.28	1.49		up	up	
CL6767.Contig2_All				−1.25	−1.37		down	down
CL6767.Contig3_All				−2.52	−3.05		down	down
CL835.Contig1_All			2.93	−2.80	−5.73	up	down	down
CL835.Contig2_All				−3.37	−3.31		down	down
CL835.Contig3_All				−2.37	−1.71		down	down
CL835.Contig4_All				−5.89	−5.70		down	down
CL835.Contig5_All			2.05	−3.30	−5.35	up	down	down
CL835.Contig6_All				−3.41	−3.94		down	down
CL835.Contig7_All			1.25	−2.26	−3.51	up	down	down
Unigene15499_All			6.55	1.10	−5.45	up	up	down
CL7407.Contig1_All	K12125	*ELF3*		−6.46	−5.48		down	down
CL8526.Contig1_All	K12124	*GI*	1.48		−1.02	up		down
CL8526.Contig2_All			2.59	−3.56	−6.16	up	down	down
CL8526.Contig3_All			1.65	1.11		up	up	
CL8526.Contig4_All				1.51			up	
CL8783.Contig1_All	K16223	*FT*	9.27	6.48	−2.78	up	up	down
CL8783.Contig2_All			6.12	3.64	−2.48	up	up	down
CL8783.Contig3_All			7.57	5.02	−2.55	up	up	down
Unigene18266_All			−5.81	−4.83		down	down	
Unigene23647_All			−5.49	−2.51		down	down	
Unigene30306_All				7.14	7.05		up	up
Unigene35531_All			−8.42	−8.44		down	down	
Unigene36039_All			−3.55	−7.15		down	down	
Unigene235_All	K12118	*CRY1*	5.47	4.40	−1.07	up	up	down
Unigene236_All			5.01	3.70	−1.31	up	up	down
CL926.Contig1_All	K12119	*CRY2*		2.81	2.28		up	up
CL926.Contig11_All				3.01	2.38		up	up
CL926.Contig2_All				2.37	3.07		up	up
CL926.Contig3_All				1.71	1.69		up	up
CL926.Contig4_All				1.12	2.06		up	up
CL926.Contig5_All			1.04	3.57	2.53	up	up	up
CL926.Contig6_All			1.22	5.43	4.20	up	up	up
CL926.Contig7_All				2.79	2.28		up	up
CL926.Contig8_All			2.09	5.79	3.70	up	up	up
Unigene2881_All			−1.41	1.19	2.60	down	up	up
Unigene21537_All	K12115	*ZTL*			1.57			up
Unigene32913_All				1.25			up	
Unigene8078_All			−1.24		1.58	down		up
Unigene8082_All				1.96			up	
Unigene8325_All				−1.95	−2.92		down	down
Unigene6219_All	K16240	*SPA1*	2.77	1.31	−1.45	up	up	down
CL773.Contig1_All	K01184	*FLC*	3.09	−6.44	−9.54	up	down	down
CL773.Contig3_All			4.41	−4.89	−9.29	up	down	down

(to be continued)

Table 1. (continued)

Gene ID	Entry	Name	log_2(T2/T1)	log_2(T3/T1)	log_2(T3/T2)	T1 vs T2	T1 vs T3	T2 vs T3
CL773.Contig4_All			4.24	−4.90	−9.14	up	down	down
CL773.Contig5_All			3.79	−6.37	−10.16	up	down	down
Unigene16702_All			3.22	−6.84	−10.07	up	down	down
Unigene16704_All				−4.77	−5.08		down	down
CL7416.Contig1_All	K09264	*FUL*	3.39	3.44		up	up	
CL7416.Contig2_All			3.51	3.34		up	up	
Unigene18421_All			4.66	−4.95		up	down	
Unigene44881_All			5.31	3.57	−1.75	up	up	down
Unigene44883_All			4.71	−7.96		up	down	
Unigene7311_All	K09264	*AP1*	7.44	12.69	5.25	up	up	up
CL8905.Contig1_All	K09260	*SOC1*	2.35	−3.64	−5.99	up	down	down
Unigene7203_All			−3.22	−7.68	−4.46	down	down	down

PsFT (AHM25242.1) was the highest, reaching 99%. The similarity between *FT* and *PhFT* (AZL87173.1) was the lowest, reaching 89%. The *PlFT* gene sequence was registered in the NCBI database with the accession number of MT249229. To study the phylogenetic relationship between *PlFT* and other species, the phylogenetic tree of *PlFT* protein and FT protein of other species is shown in Fig. 5b. The results showed that the evolutionary relationship between *PlFT* and *PsFT* was the closest, followed by *VvFT* and *HnFT*, and relatively far from *MdFT*.

Genetic transformation of *PlFT* gene in *A. thaliana*

An overexpression vector carrying *PlFT* (pCAMBIA1301-*PlFT*) was transformed into *Agrobacterium tumefaciens* strain EHA105 by the freeze-thaw method, which was transformed into *Arabidopsis* mutant *ft-10* by the floral dip method as there is currently no peony transformation system available. Mature seeds were collected and recorded as T0. The seeds of T0 generation were screened on MS medium containing hygromycin (25 mg/L). The well-developed plants as the transformed T1 generation *Arabidopsis* (Supplemental Fig. 2) were transplanted to the plug and cultured in the light incubator. After flowering and seed set, the seeds were collected and recorded as the T2 generation. The T2 generation was further seeded on hygromycin-resistant medium for screening, and the control wild-type *Col-0* and mutant *ft-10* were transplanted and seeded on MS medium without resistance. A total of 35 T2 generation transgenic *Arabidopsis* with *FT* gene of *P. lactiflora* were obtained. One complete flowering mutant *ft-10* and one wild-type *Col-0 Arabidopsis thaliana* were selected as controls. At the same time, T2 generation *PlFT* transgenic plants 4, 17, and 28 lines were chosen for GUS staining analysis (Fig. 6a). The mutant *ft-10* and wild-type *Col-0* did not exhibit blue. At the same time, the selected transgenic plants showed blue, which indicated that wild-type and mutant plants did not contain the GUS gene, while those stained with blue were transgenic plants carrying the *PlFT*.

qRT-PCR was used to detect the expression of the *PlFT* gene in *Arabidopsis thaliana*. The mutant *ft-10* and wild-type *Col-0* plants were used as the control group. Ten *PlFT* transgenic *ft-10* mutant *Arabidopsis* (3, 8, 11, 14, 18, 24, 25, 26, 27 and 33 lines) were randomly selected and analyzed. The expression levels of these plants are shown in Fig. 6b. The expression levels of the *PlFT* gene in ten transgenic lines were highest, suggesting that the *PlFT* gene was overexpressed in mutant *ft-10 Arabidopsis* driven by *ubiquitin* promoter.

The phenotypes of T2 generation transgenic *Arabidopsis* and its control are shown in Fig. 7a. *ft-10* was in the vegetative growth stage, *Col-0* was budding, while transgenic *Arabidopsis* showed early flowering phenotype. The flowering time (Fig. 7b) and rosette leaves (Fig. 7c) of transgenic *Arabidopsis* and the control were counted. The results showed that the flowering time of the mutants *ft-10* was about 70 DAE (days after emergence), the number of rosette leaves at bolting mainly was 30–35. The flowering time of wild-type *Col-0* was 57 DAE, and the number of rosette leaves at bolting was 16–23. *PlFT* complemented the late flowering phenotype of mutant *ft-10*. The flowering time of transgenic *ft-10* was about 45 DAE, and the number of rosette leaves at bolting decreased to 8–15. The difference in the flowering time between transgenic *Arabidopsis* and control was significant. The number of rosette leaves of mutant *ft-10* was the most, followed by wild-type *Col-0*, and transgenic *Arabidopsis* was the least. Because of the deletion of the *FT* gene, the flowering of mutant *ft-10* was later than that of wild-type *Col-0*. However, after the *PlFT* gene was transferred, the functional complementation was obtained. The flowering time was significantly earlier than that of wild-type, which was consistent with the statistical results of leaf disc number.

DISCUSSION

Paeonia lactiflora has become an important cut flower plant, but current research has been largely focused on physiological levels of flower bud differentiation, and there have been no transcriptome studies on flower development. However, transcriptome studies have been performed on other floral plants. For example, transcriptome analysis was conducted on the flowering process of 'Old Brush'. From which 85,663 single genes and 1,637 differentially expressed genes (DEGs) were obtained. *FRI*, *FY*, *DRM1*, *ELIP*, *COP1*, *CO* and *COL16* related to the circadian rhythm or autonomic pathway were screened[37]. A complex genetic network comprising of several coordinated flowering pathways was identified which control the developmental transition of flowering[38]. In *Arabidopsis*, more than 200 genes associated with flowering have been identified and characterized[10,39]. In this study, a total of 23 DEGs were identified to regulate peony flowering, including *CHE*, *PHYB*, *LHY*, *CO*, *PRR5*, *PRR7*,

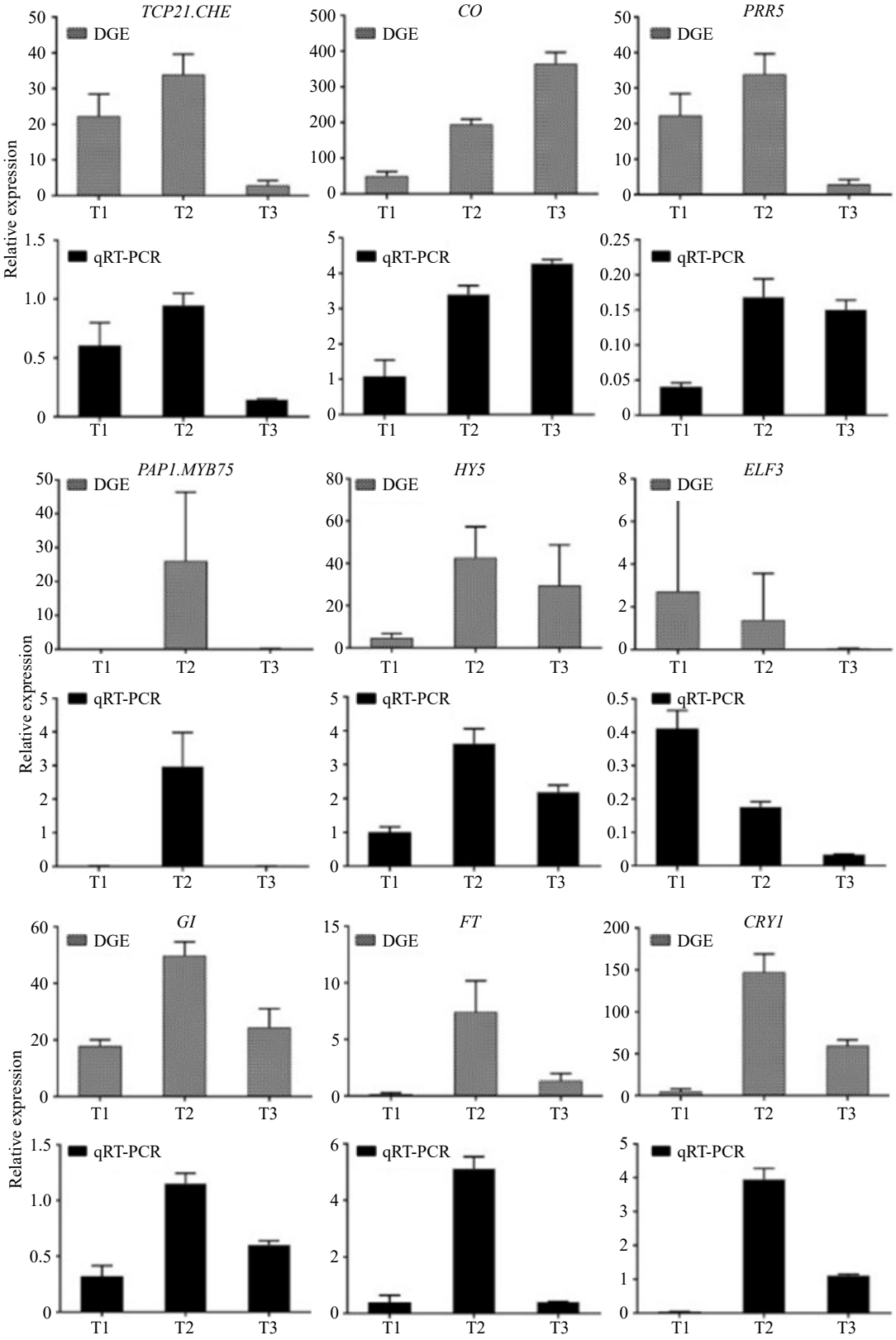

Fig. 4 qRT-PCR validations of expression levels of DEGs.

FKF1, *CDF1*, *PHYA*, *PAP1*, *HY5*, *TOC1*, *ELF3*, *GI*, *FT*, *CRY1*, *CRY2*, *ZTL*, *SPA1*, *FLC*, *FUL*, *AP1*, and *SOC1* from Illumina Hiseq platform transcriptome data. These genes were involved in various flowering pathways and regulatory networks, including the photoperiod, vernalization, and aging pathway[10,39]. This study is the first transcriptome analysis of

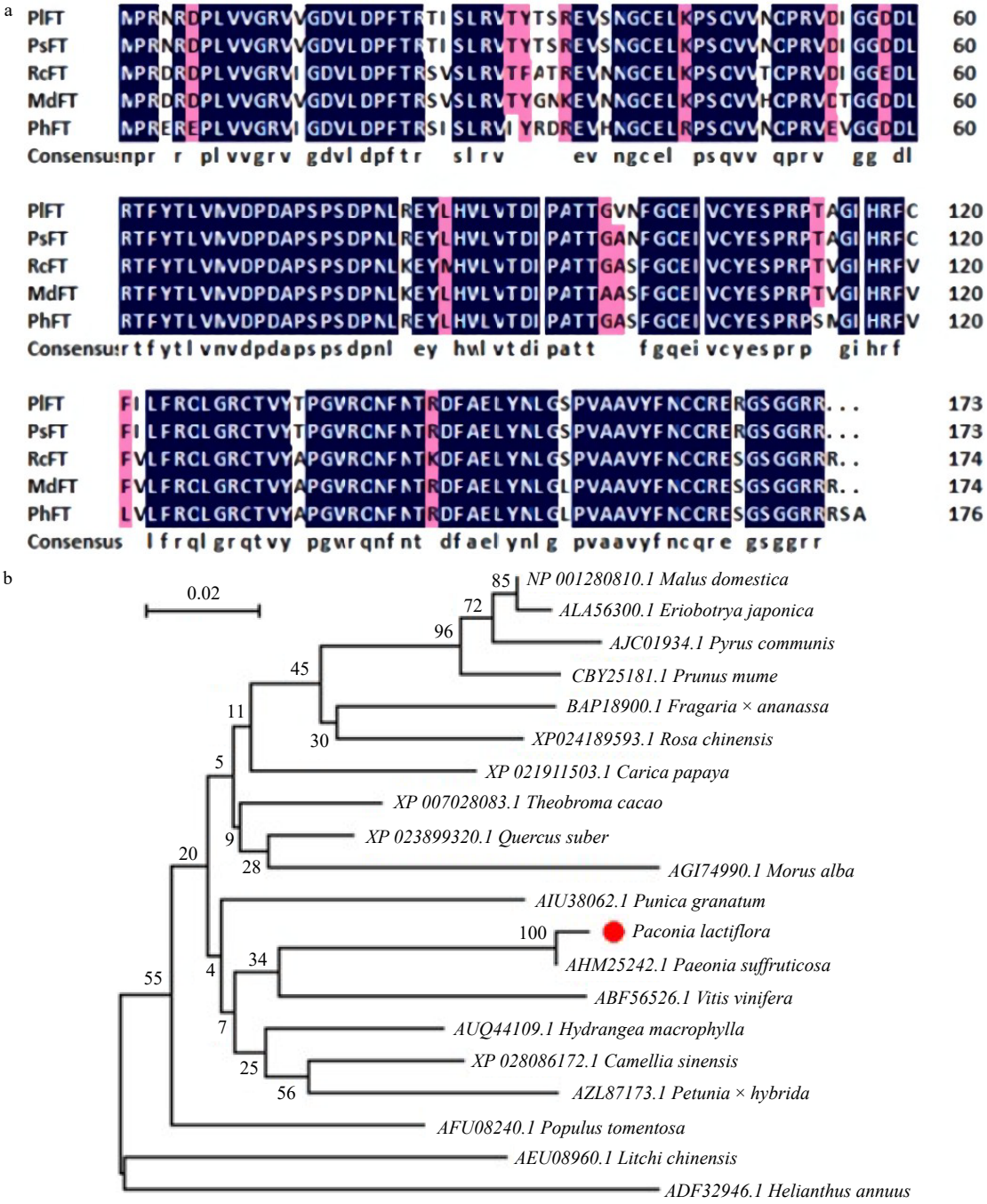

Fig. 5 Sequence analysis of *PlFT*. (a) Amino-acid comparision between *PlFT* and *FT* homologues from other species. (b) Phylogenetic tree based on the amino acid sequences from *PlFT* and other species. AHM25242.1 [*Paeonia suffruticosa*]; AFU08240.1 [*Populus tomentosa*]; BAP18900.1 [*Fragaria* × *ananassa*]; AUQ44109.1 [*Hydrangea macrophylla*]; XP_023899320.1 [*Quercus suber*]; XP_028086172.1 [*Camellia sinensis*]; AIU38062.1 [*Punica granatum*]; XP_007028083.1 [*Theobroma cacao*]; XP_024189593.1 [*Rosa chinensis*]; AGI74990.1 [*Morus alba*]; XP_021911503.1 [*Carica papaya*]; ABF56526.1 [*Vitis vinifera*]; NP_001280810.1 [*Malus domestica*]; AZL87173.1 [*Petunia* × *hybrida*]; ALA56300.1 [*Eriobotrya japonica*]; AJC01934.1 [*Pyrus communis*]; AEU08960.1 [*Litchi chinensis*]; CBY25181.1 [*Prunus mume*]; ADF32946.1 [*Helianthus annuus*].

P. lactiflora 'Dafugui' at different flowering stages, which provides a scientific basis for further screening of flowering regulation genes and for elucidating the mechanism underlying flower development of *P. lactiflora*.

It is well known that the *FT* gene plays a vital role in plant growth and development, affecting the flower opening and morphogenesis. Böhlenius et al. found that the CO/FT regulatory module controls flowering time and the short-day-induced growth cessation and bud set occurring in the autumn[40]. In tomato, *SFT* induced flowering in day-neutral tomato and altered flower morphology[41]. The *FT* orthologue in rice, the *Hd3a* gene, participated in the regulation of potato types to tuberize[42]. Moreover, overexpression of *FT* can activate the H^+-ATPase to open stomata in plants[43]. Because

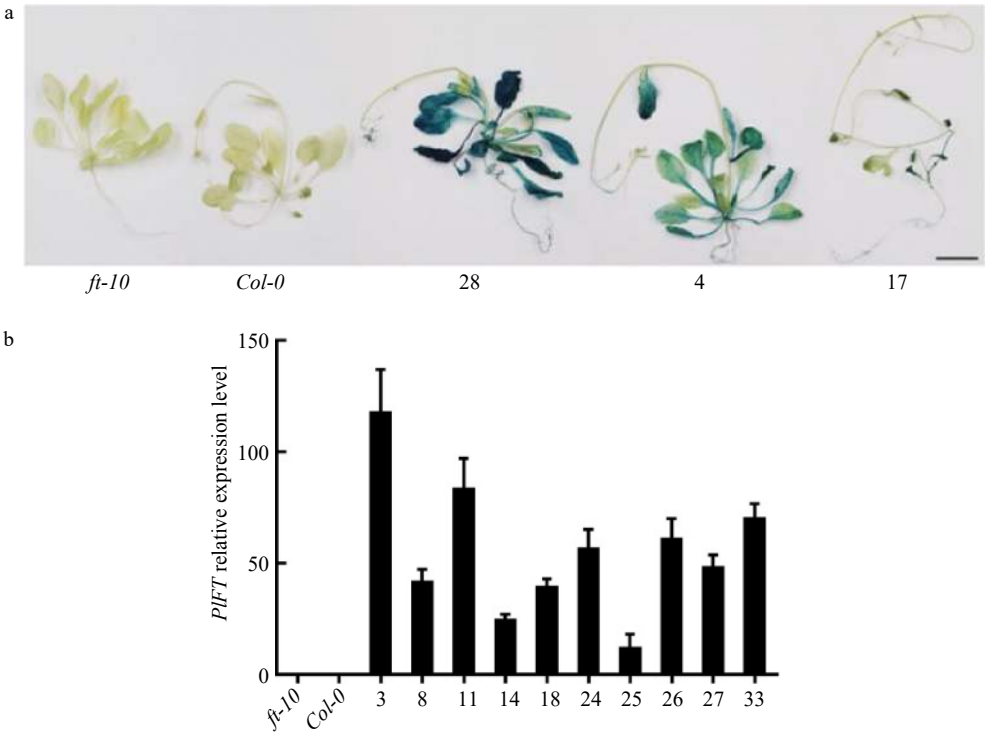

Fig. 6 Identification of the *PlFT* gene in *A. thaliana*. (a) GUS staining results of transgenic *Arabidopsis* lines with *PlFT* gene. (b) Relative expression level of target genes in transgenic lines. *ft-10* is mutant plant, *Col-0* is wild-type plant, other numbers are *PlFT* transgenic plants.

of the critical role of *FT* in plants, we chose *PlFT* as the essential flowering-related gene. Our results proved that the *PlFT* gene played an important role in regulating flowering of *P. lactiflora*.

Overexpression of *PlFT* could promote the flowering of plants, which is important for the development of *Arabidopsis*. It is suggested to use the *FT* gene to regulate the inflorescence of *P. lactiflora*. For example, overexpression of *LsFT* from lettuce (*Lactuca sativa* L.) can restore the late flowering phenotype of *ft-2* mutant *Arabidopsis*[44]. Transgenic cassava plants with *MeFT1* showed an early flowering phenotype compared with non-transgenic control. qRT-PCR analysis indicated that *MeFT1* triggered flowering by regulating downstream flower meristem recognition genes[45]. *FT* does not act alone but alongside environmental and endogenous signals that regulate downstream genes to promote flowering. Studies found that *FT* induces the transcription of *SWEET10*, which encodes a bidirectional sucrose transporter, specifically in the leaf veins. It changes the metabolism of flowering plants and is activated by long-term illumination. Ectopic expression of *SWEET10* leads to increased transcription levels of genes associated with flowering time in shoot tips, leading to early flowering of plants[46]. In the upstream of *FT*, overexpression of *CmBBX8* regulates genes expression related to photoperiod, and accelerates flowering. *CmBBX8* has been confirmed to directly target *CmFTL1* and promote the flowering of summer chrysanthemum[47]. However, not all *FT* functions were promoting flowering. Overexpression of *LlFT* and *TgFT2* in *Arabidopsis* led to the decrease of early flowering and rosette leaves. The bulb-specific role of *TgFT3* was speculated through observation and phylogenetic analysis[48]. These results indicate the complexity of flowering time regulation and the functional diversity of the *FT* gene.

In this study, the upstream and downstream genes and action sites of *PlFT* regulation in *P. lactiflora* need to be further explored. Because the *FT* gene is related to light and photoperiod, its application could make plants bloom earlier and expand the geographical scope of its production. According to relevant reports, different *GmFT2a* and *GmFT2b* haplotypes significantly affect the diversity of soybean flowering time at different latitudes[49]. The *CsFT* locus is the primary source for cucumber to adapt to high latitude, which provides an important perspective for flowering time control and latitude adaptation of cucumber and may help encourage breeding cucumber in the cold temperate zone[50]. Thus, the multi-function of *FT* could make it a valuable resource for regulating the flowering time of *P. lactiflora*. The overexpression of *PlFT* could accelerated the flowering of *A. thaliana* and reduced the rosette leaves. *PlFT* function study will help us to understand the molecular mechanism of flowering in *P. lactiflora* and provide important resources for genetic improvement of *P. lactiflora* as one of the important cut flower plants.

MATERIALS & METHODS

Plant materials and sample preparation

Plants of *Paeonia lactiflora* 'Dafugui' had cultivated for three years in the germplasm repository of the Horticulture and Plant Protection College, Yangzhou University, Jiangsu Province, P.R. China (32°39′N, 119°42′E). Shoot apical meristem (T1), flower buds (T2) and flowers (T3) were

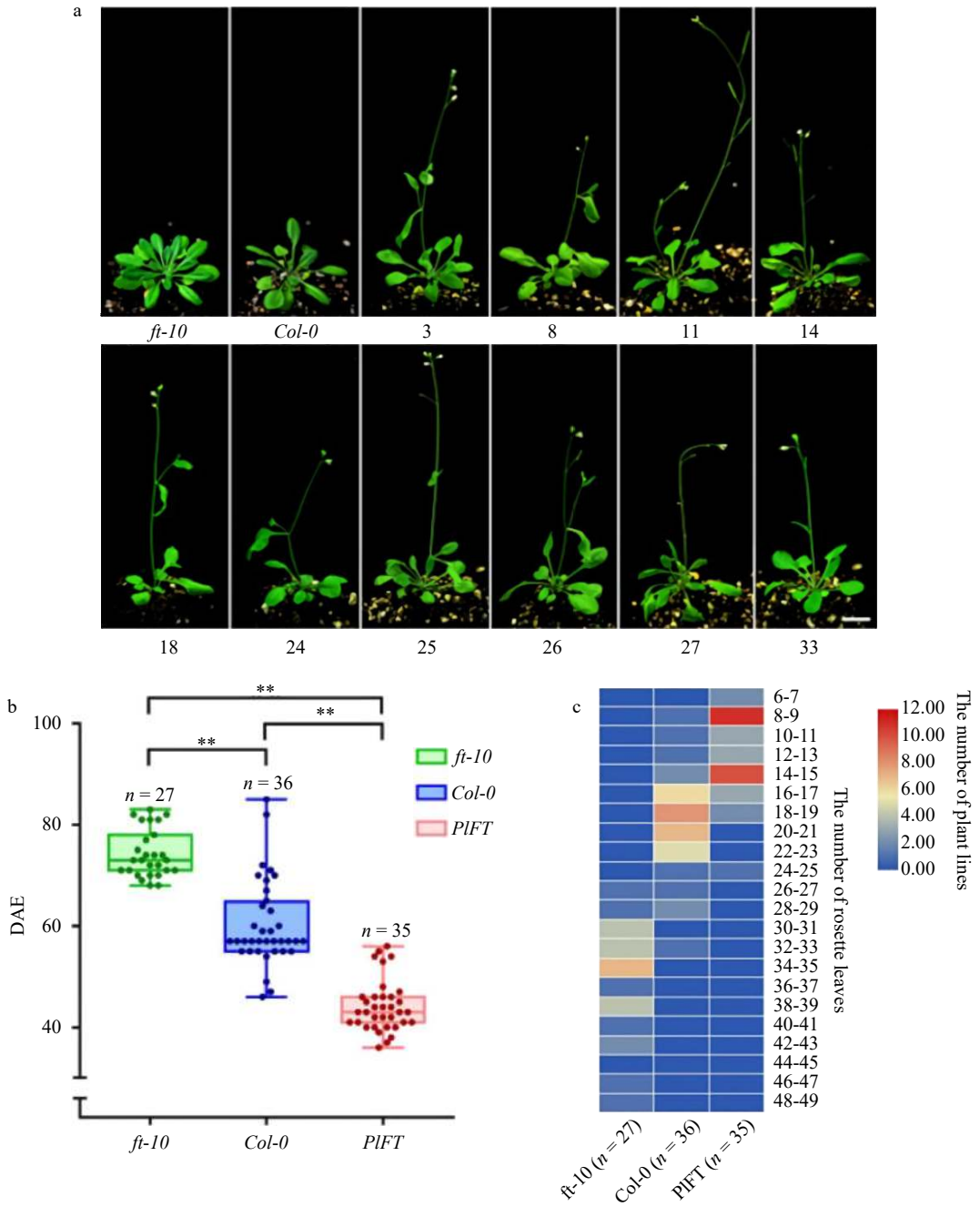

Fig. 7 Phenotypic analysis of the *PlFT* gene in *A. thaliana*. (a) Phenotype of transgenic *Arabidopsis* (Scale = 1 cm). (b) Flowering time of *Arabidopsis* DAE in the figure indicates the days after emergence and indicate significant differences ($P < 0.01$). (c) Heat map of the leaves of the rosette of *Arabidopsis thaliana*.

separately collected on June 17, 2018, March 9, 2019 and May 9, 2019. They were stored at −80 °C for RNA extraction. RNA was separately extracted for these samples using the Mini BEST RNA Extraction Kit (TaKaRa), and RNA samples was checked using Nanodrop 2000C (Thermo Scientific).

cDNA library construction and sequencing

After total RNA was extracted from 'Dafugui', the sampling time points included three stages with three biological repeats in each step. A total of nine samples were used to construct cDNA library and de novo sequencing. The total RNA was performed by mRNA enrichment or rRNA removal. The obtained mRNA was fragmented by adding an appropriate amount of interruption reagent under high temperatures. A strand of cDNA was synthesized by using the interrupted mRNA as a template, the two-strand cDNA was then synthesized by configuring a two-strand synthesis reaction system. The purified cDNA was recovered, and the sticky end was repaired. The 3 'end of the cDNA was added with a base 'A' and connected to the connector. The fragment size was then selected, and PCR amplification was performed. Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR

system were used to detect the constructed library. Finally, the RNA was sequenced after qualification.

Sequencing data filtering and assembly

The filtering software SOAPnuke (v1.4.0) and trimmatic (v0.36) were used for statistics and filtering respectively, and RSEM (v1.2.8)[51] was used to calculate the expression levels of genes and transcripts.

Trinity (v2.0.6) software (https://github.com/trinityrnaseq/trinityrnaseq/wiki) was used for de novo assembly of clean reads. Tgicl clustered the transcript to obtain Unigene. The Unigene is divided into two parts. One is clusters, which is the result of further redundancy. To study multiple samples, we used Tgicl to cluster the Unigenes of each sample again to obtain the final Unigene for subsequent analysis.

Functional annotation and classification

Seven functional databases (KEGG, NR, GO, NT, SwissProt, Pfam and KOG) were annotated to obtain the protein function annotation and metabolic pathway annotation of Unigene. Blastn is used to annotate Unigene in NT, and Blastx annotates Unigene in NR, KOG, KEGG and Swiss-Prot. Blast2Go and NR are used to annotate GO, and InterProScan5 is used to annotate InterPro.

Analysis of differentially expressed genes (DEGs)

The FPKM values of different genes in each comparison group were clustered. According to the detection results of DEGs, hierarchical clustering analysis was performed by the heat map function in R software. According to GO annotation and official classification, the results of differential gene detection were classified into parts. According to KEGG annotation results and official type, the results of differential gene detection were classified into biological pathways. At the same time, the hyper function in RESM software was used for enrichment analysis, then, P-value was calculated and was corrected by FDR (false discovery rate). DEGs analyzed the data of KEGG pathway in different periods. $\log_2$ (FPKM of treatment group /control group) > 0 indicates that the gene expression of the treatment group is up-regulated compared with that of the control group, and < 0 indicates down-regulation.

Quantitative real-time PCR analysis

Total RNA from all samples in *P. lactiflora* extracted by a MiniBEST Plant RNA Extraction Kit (TaKaRa, Japan) was used to synthesize cDNA by PrimeScript RT reagent Kit With gDNA Eraser (TaKaRa, Japan)[52] to anlayze expression levels with a BIO-RAD CFX Connect Optics Module (Bio-Rad, Des Plaines, IL, U.S.A.). The $2^{-\Delta\Delta Ct}$ comparative threshold cycle (C_t) method was referred to calculate their values. 12.5 μL 2 × SYBR Premix Ex Taq, 2 μL cDNA solution, 2 μL mix solution of primers, and 8.5 μL ddH_2O in a final volume of 25 μL are the system to perform qRT-PCR. The amplification conditions are 95 °C for 30 s, 40 cycles at 95 °C for 5 s, 52 °C for 30 s, and 72 °C for 30 s.

qRT-PCR was performed to analyze the expression levels of flowering-related DEGs and to detect the expression of the *PlFT* gene in *Arabidopsis thaliana*. All used primers are listed in Supplemental Tables 4 and 5.

Sequence analysis of one flowering-related gene

Total RNA was extracted from the fresh leaves of 'Dafugui' with a Plant RNA kit (TaKaRa, Japan). According to PrimeScript® RT reagent Kit with gDNA eraser (Perfect Real Time), RNA was reserved into cDNA. Using 5' and 3' end primers synthesized commercially (Genery, China) were designed based on the ORF sequence of the full-length sequence of DEGs FT (gene ID CL8783.Contig2_All) in transcriptome data. PCR reaction was as follows: one cycle of 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min; and one cycle of 72 °C for 10 min. After testing by 1 % (w/v) agarose gel electrophoresis, the PCR products were cloned into the pClone007 Vector and sequenced. All gene-specific primers (Supplemental Table 5) were designed by Primer Premier 5.0. DNAMAN 7.0.2 was used to assemble multiple alignments of protein sequences of gene ID CL8783.Contig2_All with those from other species. A Neighbor-Joining phylogenetic tree was generated with MEGA 7.0, using the Poisson correction method and 1000 bootstraps.

Expression vector construction

The recombinant plant transgenic vector was based on the obtained full-length sequence of the target gene, combined with the restriction site of the binary expression vector pCAMBIA1301 UbiNOS (Supplemental Fig. 3) constructed by single fragment homologous recombination. The *Sac* I and *Kpn* I restriction sites on the polyclonal site of pCAMBIA1301 were used to double cleave the vector (Supplemental Fig. 4). At the same time, the coding region of the *PlFT* gene was amplified with primers (Supplemental Table 5) containing a 15-20 bp sequence of linearization vector (Supplemental Fig. 5). The recombinant plasmid pCAMBIA1301-*PlFT* was constructed by ligating the target fragment to the vector with Exnase.

Transformation of flowering-related *PlFT* gene into *A. thaliana*

The expression vector pCAMBIA1301-*PlFT* plasmids were used for the transformation of competent cells of Agrobacterium tumefaciens strain EHA105. *Arabidopsis Col-0* plants were transformed using the floral-dip method[53]. The inflorescence of *A. thaliana* was soaked with 1/2 MS infection liquid with transformed *Agrobacterium* for 1 min and then cultivated under dark conditions for 12 h. The seeds collected were recorded as T0 (the first generation) seeds. Transgenic seeds were all screened on MS medium containing 25 mg L^{-1} hygromycin (Hyg).

GUS staining

Using the GUS staining method, the T2 generation homozygous transgenic plants were selected, and the wild-type plants in the same period were used as the negative control. The appropriate amount of GUS staining solution was added to the penicillin bottle to completely immerse the tissue. After incubation at 37 °C for 1–24 h, blue gradually appeared with the prolongation of incubation time. When the expression level was high, the active site appeared blue. The sample was then immersed in 70 % ethanol for 1–3 h until the chlorophyll of the sample was removed.

ACKNOWLEDGMENTS

This work was supported by funding from the National Natural Science Foundation of China (31600564), Modern

Agricultural Industrial Technology System in Jiangsu Province (JATS [2020]436), the Natural Science Fund of Jiangsu Province (BK20160460), the program of key members of Yangzhou University outstanding young teachers.

Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary Information accompanies this paper at (http://www.maxapress.com/article/doi/10.48130/OPR-2021-0007)

Dates

Received 6 April 2021; Accepted 13 July 2021; Published online 29 July 2021

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