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Isolation of *FLOWERING LOCUS T* Gene and Analysis of Periodic Expression Level in *Cymbidium kanran*

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Abstract *Cymbidium kanran*, as one of the five most famous Chinese *Cymbidium*, has high ornamental value, and it's principal flowering period varies from October to December. In order to study the flowering-regulation, the *FLOWERING LOCUS T* Gene (*FT*) of *C. kanran* sequence was amplified, and tissue-specific expression of the gene was analyzed in the whole flowering period. The results show: (1) The ORF sequence of the *FT* is 531 bp in length and the gene encodes 177 amino acids, and the sequence is highly homologous to the reported cymbidium such as *C. ensifolium* and *C. goeringii*; (2) During August to September, the expression of *FT* in the leaves of flowering *C. kanran* was 1.3 times higher than that of unflowered group, but there was no significant difference in the expression level of this gene at the other months; (3) During August to December, the expression level of *FT* in the flowering *C. kanran* pseudobulbs were 1.56, 2.46, 3.10, 2.70, and 1.42 times than that of unflowered group, respectively; (4) During flowering, the relative expression level of *FT* in pseudobulb of flowering *C. kanran* was at least 15.80 times higher than that in leaves; (5) The expression of *FT* in pseudobulbs were higher than that of flower stem and perianths, and slightly lower than that of primary flower bud. The above results fully show that there is an important correlation between the expression pattern of *FT* of the leaves and pseudobulbs in a specific period, which provides theoretical significance and application value for future research in the flowering-regulation of *C. kanran* and artificial flowering experiments.
Keywords *Cymbidium kanran*; Flowering period; *FLOWERING LOCUS T* gene; Expression patterns

Hanlan (*Cymbidium kanran*) is a group of terrestrial orchids of *Cymbidium* genus in the family of Orchidaceae. It is mainly distributed in Zhejiang, Fujian, Taiwan, Guangdong and other places in China. It is one of the five most famous Chinese *Cymbidium*. The plant height is about 20~40 cm, and the flower diameter is about 3~6 cm. *Cymbidium kanran* is elegant and pleasant, with excellent humanistic appreciation value (Figure 1). Through investigation of its growth, it was found that the flowering mode of the *Cymbidium kanran* was different from that of other Chinese *Cymbidium*: (1) The buds of the *Cymbidium kanran* differentiated from August to October, and the flower buds blossomed directly after differentiation. Unlike Chunlan (*C. goeringii*), Huilan (*C. faberi*) and Molan (*C. sinense*), there was no dormancy of flower buds. (2) The flowering period was mainly from October to December, which happened to be at the stage of light weakening and temperature decreasing compared with other five Chinese *Cymbidium*; (3) The phenomenon of irregular flowering of different single plant of *Cymbidium kanran*, sometimes can be seen in summer. In conclusion, the flowering of the *Cymbidium kanran* cannot be simply attributed to a single environment or autonomous flowering factors, and there may be a more complex regulatory relationship.

Flowering physiology has always been one of the hot topics in botany research. In long-term agricultural production and life, it has been observed that the length of light, temperature and external hormone application have very important effects on plant flowering. By studying the flowering regulation model of *Arabidopsis thaliana*, it was found that (Figure 2) the function of FT (Flowering locus T) protein is mainly to promote flower bud differentiation (Kardailsky et al., 1999; Corbesier et al., 2007). Four important flowering regulatory pathways (photoperiod pathway, GA pathway, vernalization pathway and autonomic flowering pathway) require

transcription factors to activate the expression of *FT* genes to promote flowering of *Arabidopsis thaliana* (Kinmonth-Schultz et al., 2016; Wang et al., 2020). Long-day illumination can activate *BvFT2* gene expression of long-day flowering sugarbeet (*Beta vulgaris*), but can not activate the expression of short-day sugarbeet related genes (Štorchová et al., 2019). The *PdFT* gene of peony (*Paeonia suffruticosa*) is related to its floral organ development (Zhu et al., 2014). Studies on flowering regulation in rice (*Oryza sativa*) (Kojima et al., 2002; Ogiso-Tanaka et al., 2013) and sorghum (*Sorghum bicolor*) (Nuñez and Yamada, 2017) found that RFT protein and Hd3a protein work together to promote flowering, and *Hd3a* gene and *Arabidopsis thaliana ELF3* gene belong to *FT* homologous genes. In addition, a large number of *FT* homologous genes have been cloned in plants, such as Jili (*Tribulus terrestris*) (Putterill et al., 2013), wheat (*Triticum aestivum*) (Yan et al., 2006), chrysanthemum (*Dendranthema morifolium*) (Pan et al., 2010), camellia (*Camellia japonica*) (Sun et al, 2014; Fan et al, 2015; Lei et al, 2017) and cotton (*Gossypium*) (Guo et al., 2015). Further studies showed that over-expression of *RFT1* in late flowering rice could transform it into early flowering rice (Pasriga et al., 2019). Early flowering chrysanthemums can also be cultivated by expressing *FT* genes in chrysanthemums (Jiang et al., 2010). In the tomato (*Solanum lycopersicum*) (Li et al., 2009), it was also achieved by heterogeneously expressing the *MdFT* genes of *Arabidopsis thaliana* and apple (*Malus domestica*). These results indicate that the expression of *FT* gene is significantly related to the regulation of photoperiod, hormone, temperature and autonomous flowering pattern of plants.

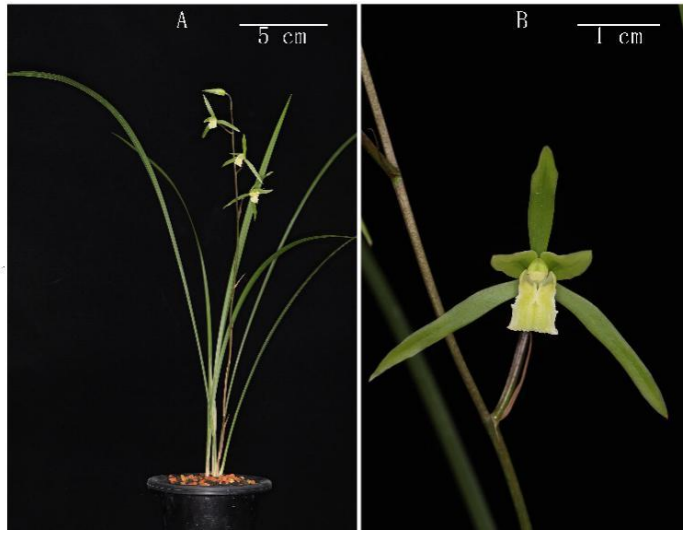

Figure 1 The *Cymbidium kanran* owns the different colors and patterns
Note: A: The plant type of *Cymbidium kanran*; B: The flower type of *Cymbidium kanran*

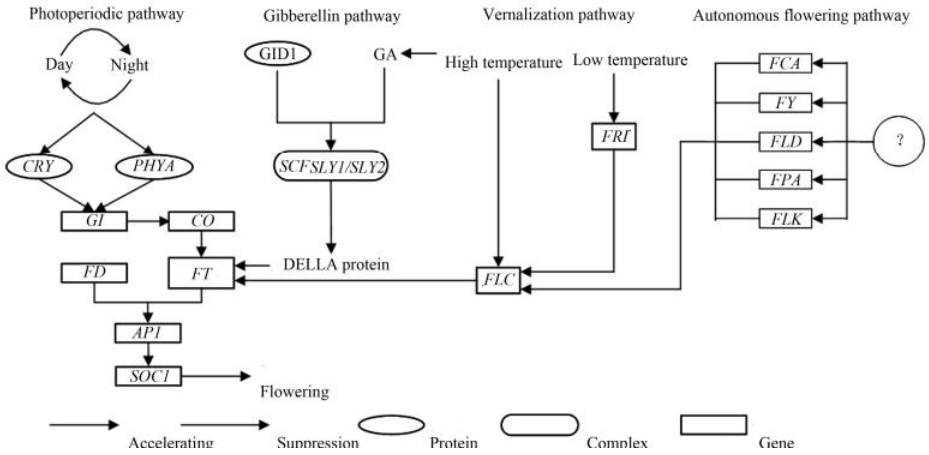

Figure 2 The Molecular mechanism of flowering regulation of *Arabidopsis thaliana*

The *FT* gene transcripts of *C. ensifolium* (HM803115.1), *C. sinense* (HM120862.1), *C. faberi* (HQ164434.1), *C. goeringii* (HM120863.1 and HM106985.1), *Oncidium hybridum* (KJ909968.1; EU583502.1) and *Phalaenopsis aphrodite* (JK720571.1) have been found in the existing NCBI database. Among them, *FT* genes of *C. ensifolium* and *C. goeringii* have been verified to promote flower bud formation and flowering in advance through *Arabidopsis thaliana* and *Nicotiana tabacum* transformation systems (Huang et al., 2012; Xiang et al., 2012; Li et al., 2013; Sun et al., 2013).

As an important member of the five most famous Chinese *Cymbidium*, there are few reports on its flowering mechanism. As ornamental flowers, the most important ornamental value is mainly reflected in the color and pattern. At present, the breeding method of *Cymbidium kanran* is mainly through hybridization and then sterile seeding and cultivation to seedlings, and the plants bloom after 5 years, which leads to a relatively long time limit for the selection of excellent varieties of *Cymbidium kanran*, making the flowering period the primary factor restricting the breeding of new varieties of *Cymbidium kanran* and industrial promotion. If we can break through the bottleneck of the flowering period of *Cymbidium kanran*, we can screen the varieties in advance and shorten the existing breeding cycle, which is of great significance to enhance the ornamental value and economic value of *Cymbidium kanran*. Combined with the existing research on flowering regulation of plants and the *FT* gene research reports of *C. ensifolium* and *C. sinense* in the early stage, this study focused on *FT* gene, cloned the *FT* gene of *Cymbidium kanran*, and studied the *FT* gene cycle and tissue-specific expression level of flowering and non-flowering *Cymbidium kanran* in the current year, in order to provide an important theoretical basis for shortening the breeding cycle of *Cymbidium kanran* in the future and establishing a complete flowering regulation mechanism model of *Cymbidium kanran* and breeding application.

1 Results and Analysis

1.1 *FT* gene amplification and molecular biological analysis of *Cymbidium kanran*

A 622 bp DNA fragment was obtained from the petal tissue of many *Cymbidium kanran* plants by PCR amplification. By sequencing analysis, the fragment contained 531 bp ORF sequence, which was more than 99% similar to the reported *FT* gene sequences of *C. ensifolium* (HM803115.1), *C. sinense* (HM120862.1), *C. faberi* (HQ164434.1), and *C. goeringii* (HM120863.1 and HM106985.1) through NCBI database comparison, which was 3 bp shorter than that of *Oncidium hybridum* (KJ909968.1; EU583502.1) and *Phalaenopsis aphrodite* (JK720571.1). The results showed that the amplified sequence was the *FT* gene sequence of *Cymbidium kanran* (Figure 3).

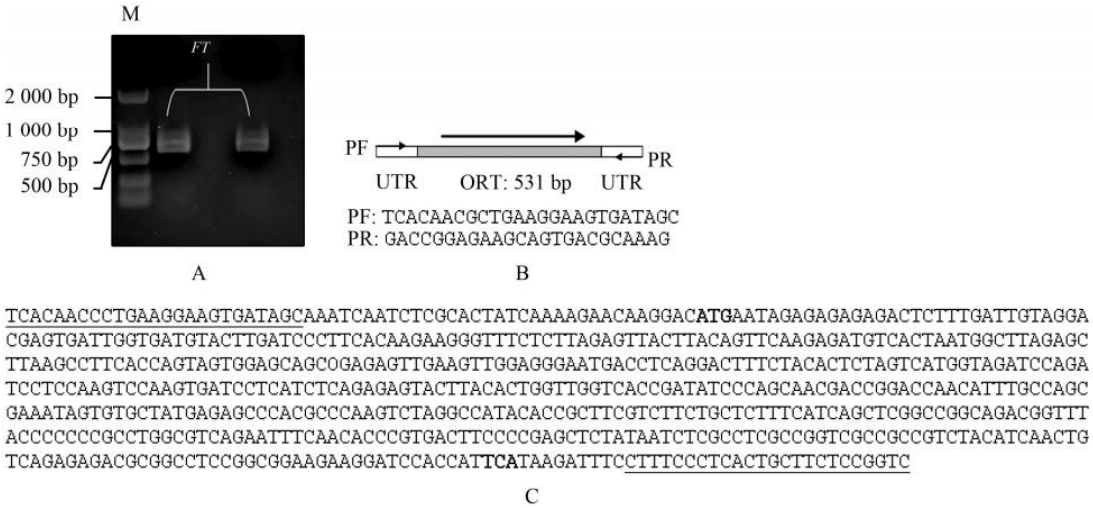

Figure 3 The *FT* gene sequence of *C. kanran*

Note: A: Amplification of *FT* gene in the petals of *C. kanran* by PCR; B: The amplified the *FT* gene structure and the primers used; C: The full-length sequence of *FT* gene amplified contains 5'and 3'UTR regions; M: The fragment lengths are 2 000, 1 000, 750, 500, 200 and 100 bp in sequence

Through clustering analysis of *FT* gene sequences of *Cymbidium kanran* and 24 other plants, the results showed that *Cymbidium kanran* was highly related to *C. ensifolium*, *C. goeringii*, *C. faberi* and *C. sinense* in the same genus, followed by *Dendrobium nobile* and *Oncidium hybridum* in the same family. The 177 amino acid sequences corresponding to *FT* gene of *Cymbidium kanran* were highly similar to those of *C. ensifolium*, *C. goeringii*, *C. faberi* and *C. sinense* in the same genus (Figure 4). According to the above results, it can be seen that the *FT* gene sequence in *Cymbidium* has a high conserved type. Based on the previous reports on the function of *FT* gene sequences of *C. ensifolium* and *C. goeringii*, it can be concluded that the *FT* gene amplified in this paper may also play an important role in the regulation and bud induction of *Cymbidium kanran*.

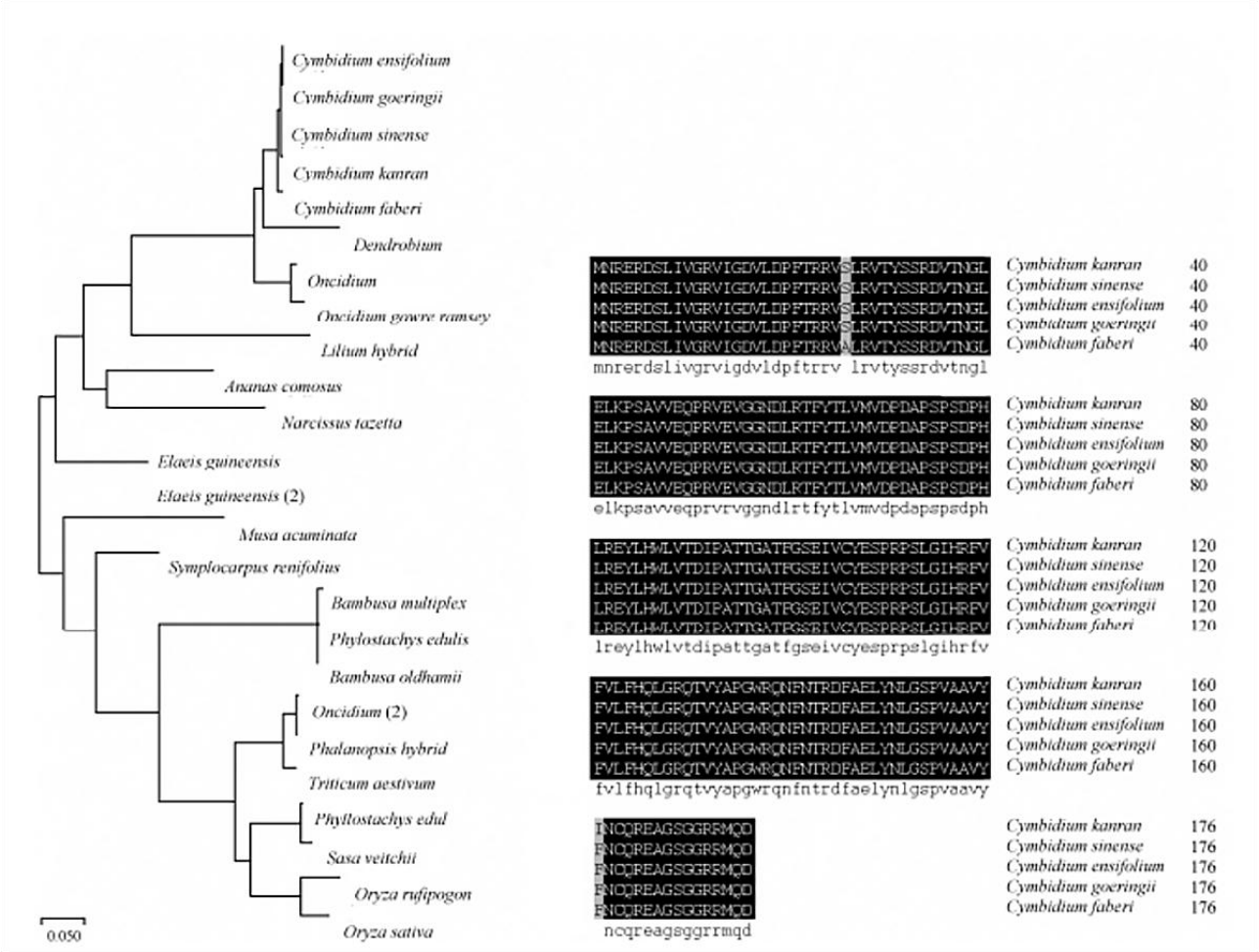

Figure 4 The *C. kanran FT* gene sequence gene clustering and amino acid sequence analysis

1.2 Analysis of relative expression level of *FT* gene in leaves

By monitoring the *FT* gene expression levels in the leaves and pseudobulbs of the *Cymbidium kanran* in one flowering cycle, we could see that there were significant differences between the flowering group and the non-flowering group in the beginning to the end of the flowering period.

From January to February of the next year, the expression level of *FT* gene in the leaves of the non-flowering group fluctuated around 1.000, and the difference between the data groups was not significant. There was no significant difference between the flowering group and the non-flowering group in gene expression data from January to July and from October to February of the next year ($P>0.5$). However, in August and September, the critical period of bud differentiation of *Cymbidium kanran*, the *FT* gene expression level in the leaves of the flowering group was significantly 1.3 times higher than that of the non-flowering group, and the expression curve showed a trend of first rising and then decreasing (Figure 5). According to the regulation of *FT* gene expression in

rice and *Arabidopsis thaliana*, *FT* gene is usually expressed in leaves in response to photoperiod, and FT protein is transported to the stem to promote flower bud differentiation. The up-regulated expression of *FT* gene in the flowering group and the expression of *FT* gene in the non-flowering group are also basically consistent with the previous report (Zhang et al., 2013).

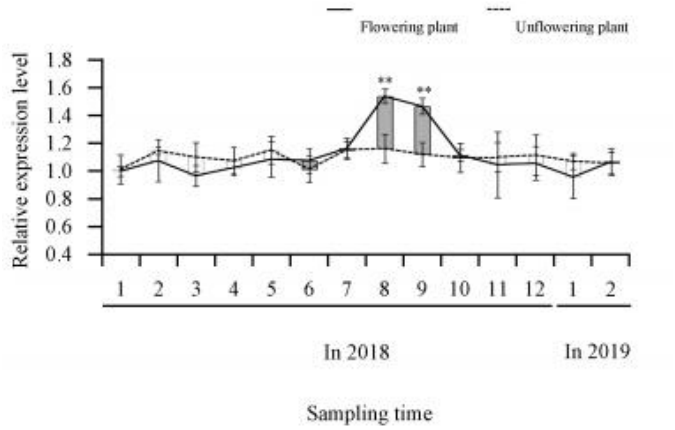

Figure 5 The relative expression level of *C. kanran FT* gene of leaves in one year
Note: ** $P<0.01$

1.3 Analysis of relative expression level of *FT* gene in pseudobulb

Pseudobulb is the most important nutritive tissue of cold orchid, and its leaves, roots and flowers grow from it. Complete plants were damaged after sampling. Before flower bud differentiation in January to July, the non-flowering group and flowering group shared one set of data. In August, after the flower bud differentiation of pseudobulb, *FT* gene quantitative detection was performed on flowering group and non-flowering group respectively, and the results showed that: During flower bud differentiation and flowering, *FT* gene expression levels in pseudobulbs in flowering group were extremely high, and the average expression levels from August to December were 1.56, 2.46, 3.10, 2.70 and 1.42 times of those in non-flowering group, respectively (Figure 6). By comparing the curves between the two groups, it can be seen that the *FT* gene expression level in the pseudobulbs of the flowering group is very high, which is consistent with the previous report that *FT* gene plays an important role in the regulation of plant flowering. Compared with leaves, high *FT* gene expression in pseudobulb can provide better molecular catalysis for flower bud differentiation and growth. It also showed that pseudobulbs were the most important tissues in the vegetative and reproductive growth of the *Cymbidium kanran*, and many regulatory mechanisms related to the physiological growth of the *Cymbidium kanran* were mainly reflected in pseudobulbs.

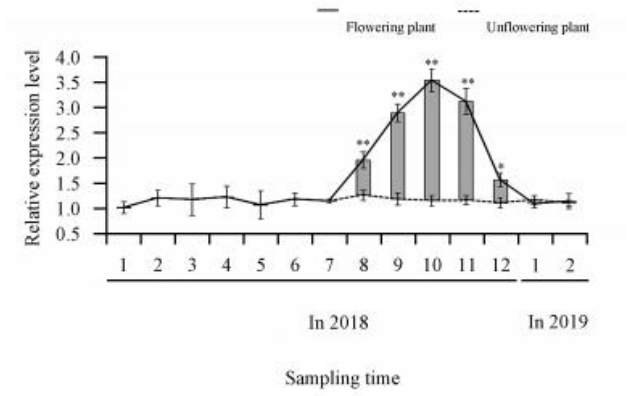

Figure 6 The relative expression level of *C. kanran FT* gene of pseudo bulbs in one year
Note: ** $P<0.01$, *$0.5<P<0.01$

1.4 Changes of relative expression level of FT gene in flowering *Cymbidium kanran* tissue

FT gene expression analysis was carried out on the leaves, pseudobulbs and flower buds of flowering group *Cymbidium kanran* during flowering. The results showed that the expression level in leaves was the lowest among all vegetative organs, the expression level in pseudobulb was 11.3 times higher than that in leaves, and the expression level in pseudobulb was 37.9 times higher than that in leaves. Therefore, *FT* gene was slightly expressed in leaves, but greatly expressed in pseudobulb after the flowering of *Cymbidium kanran*. In floral organs, pseudobulbs could be expressed in the whole flower bud and perianth, and the expression rate was at least 15.80 times compared with leaves. At the early stage of flower bud growth in September, the total expression level of *FT* gene in the whole flower bud was slightly higher than that in the flower stem, with no significant difference. In October, flower buds gradually differentiated into flower buds and flower stems. At this time, the *FT* gene expression level in flower stems and flower buds was significantly lower than that in pseudobulbs, while the *FT* gene expression level in flower buds was slightly higher than that in flower stems, with no significant difference. With the development of petals in November, the expression level of FT gene in pseudobulb decreased, and was slightly higher than that in flower stem and significantly higher than that in petals, while FT gene in petals was significantly lower than that in flower stem and pseudobulb (Figure 7). By comparing the difference of *FT* gene expression levels in vegetative organs and floral organs, it can be seen that the relative expression level of *FT* gene in the leaves of *Cymbidium kanran* is very low, while the relative expression level in the pseudobulb and floral organs is very high. This result is similar to the previous report on *C. faberi* (Sun et al., 2013). These results suggest that *FT* gene plays an important role in regulating flower bud differentiation and flower organ growth on pseudobulb.

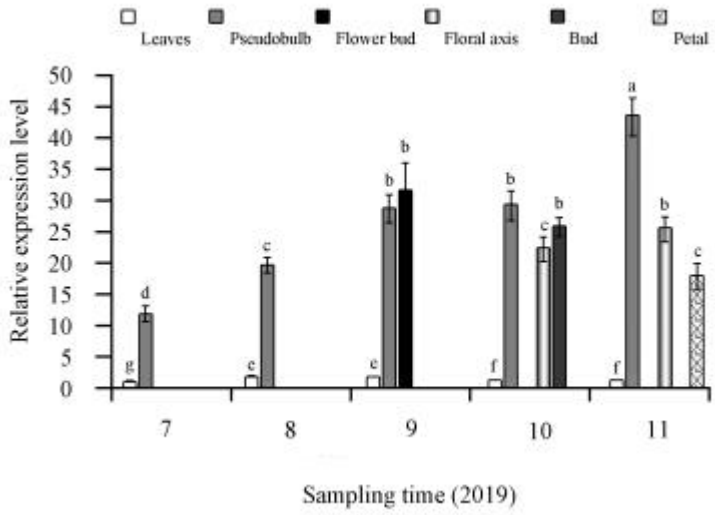

Figure 7 The relative expression level of flowering *C. kanran FT* gene of tissues in flowering phase

2 Discussion

FT gene has been widely studied as a popular flowering regulation gene in recent years. The study of molecular level of *Cymbidium kanran* stage through *FT* gene of *Cymbidium kanran* stage has important theoretical guiding significance to further reveal the regulation mechanism of *Cymbidium kanran* stage. Compared with previous reports on flowering regulation of orchids, this study not only amplified *FT* genes of orchids and analyzed molecular information, but also studied the expression levels of *FT* genes in leaves and pseudobulbs throughout the flowering cycle of orchids. The results also fully demonstrated that flowering regulation of orchids is positively correlated with *FT* gene expression. Compared with the non-flowering group of the same year, it was found for the first time that *FT* gene was up-regulated in both leaves and pseudobulbs during the flowering period, but the increment of expression was different. This indicated that the primary condition for promoting the flowering of *Cymbidium kanran* was to activate the expression of *FT* gene in pseudobulbs, so that the new shoots

shifted from vegetative growth to reproductive growth. In the past, it was widely believed that under photoperiodic regulation, FT protein was synthesized in leaves and transported to meristem to promote flower bud differentiation. However, the regulation of *Cymbidium kanran* was obviously inconsistent with previous classical studies. Although changes in *FT* gene expression have been detected in leaves, the expression level of *FT* gene in flowering leaves is much lower than that in pseudobulbs. Therefore, how *FT* gene is activated and expressed in pseudobulb is the key to study the molecular mechanism of the regulation of *Cymbidium kanran* stage, and the biological function of *Cymbidium kanran* leaves in the regulation of flowering stage also needs to be further revealed. At present, our research team has started to conduct relevant studies on *FT* gene promoter sequence and upstream genes *CONSTANS*, *FLOWERING LOCUS D*, *FLOWERING LOCUS C* etc., in order to understand how *FT* gene activates high expression in pseudobulbs of *Cymbidium kanran*. On this basis, combined with the environmental factors, we further systematically elucidate the molecular mechanism of the flowering regulation of the *Cymbidium kanran*.

3 Materials and Methods

3.1 Pretreatment and sample collection of *Cymbidium kanran*

The experiment was carried out from December 2017 to February 2019, which was the complete flowering period of the *Cymbidium kanran*. All *Cymbidium kanran* are flowering *Cymbidium kanran* in 2017, growing healthily. They are all planted in the experimental greenhouse of Zhejiang Subtropical Crop Research Institute, and grow naturally.

Leaves of *Cymbidium kanran* were collected once a month from 9: 00 to 11: 00 am on Monday of the same month, and 3 biological replicates were collected within a month and marked for classification after flowering. Pseudobulbs were collected from 9: 00 to 11: 00 am on the first Monday of every month. Use a sharp scalpel to cut off the surface of the pseudobulb at the cuticle of the stem node (flower buds need to be removed during flowering). The cuticle of the pseudobulb cut off should contain as little starch and polysaccharide as possible for liquid nitrogen grinding and extraction of high quality RNA. Three biological replicates were taken each time. After flower bud differentiation, samples were taken respectively according to flowering group and non-flowering group. After flowering (the outer three petals were basically in the same plane), tepals were taken respectively (a single flower with six petals inside and outside were taken for mixed detection), and 3 biological replicates were taken each time. The sampling times and repetitions (Table 1). All samples were frozen in liquid nitrogen and stored at -80 ℃. Nucleic acid was extracted in batches.

Table 1 Tissues and organs from which RNA is extracted at different stages

Growth stage		Dormant period				Leaf bud growth period				Flower bud differentiation and flowering period				Dormant period	
Month		1	2	3	4	5	6	7	8	9	10	11	12	1	2
Sampling site	Leaf	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √	√ √ √
	Pseudobulb	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	Floral organ	-	-	-	-	-	-	-	-	√ √ √	√ √ √	√ √ √	-	-	-

Note: "√" indicates the number of samples

3.2 RNA extraction and cDNA library construction of *Cymbidium kanran*

RNA extraction kit (RN09-EASYspin plant RNA rapid extraction kit, Beijing Aidlab Biotechnologies Co., Ltd.) was used to extract total RNA from the covers, leaves (complete appearance and free from diseases and pests) and pseudobulbs of *Cymbidium kanran*, respectively. After ensuring purity, reverse transcription (PrimeScript™ II 1st Strand cDNA Synthesis Kit, TaKaRa) was performed to complete the construction of cDNA library.

3.3 *FT* gene amplification of *Cymbidium kanran*

According to the sequences reported by NCBI of *C. ensifolium* (HM803115.1) and *C. goeringii* (HM120863.1 and HM106985.1), Bioedit software was used to compare the second-generation transcriptome database of *Cymbidium kanran* organs previously completed by our research group, and part of *FT* gene sequences of *Cymbidium kanran* were extracted, and primers were designed for amplification (Table 1). High fidelity DNA polymerase was used for PCR reaction. The amount of reagent and template (cDNA obtained from petal RNA of *Cymbidium kanran* with different flower colors) was added according to the instructions. Reaction conditions: predenaturation at 95 °C for 1 min, denaturation at 98 °C for 10 s, annealing at 56 °C for 15 s, extension at 72 °C for 1 min, a total of 30 cycles, then fully extended at 72 °C for 10 min and stored at -20 °C.

3.4 Fluorescence quantitative PCR detection

According to the sequencing results, fluorescence quantitative primers for *FT* gene of *Cymbidium kanran* were designed (Table 2). *ACTIN* was the internal reference gene, and fluorescence quantitative PCR (QuantStudioTM 7 Flex, Applied Biosystems) was performed using a special fluorescence quantitative kit (TB Green® Premix Ex Taq™ II (Tli RNaseH Plus), Bulk, TaKaRa). The standard procedure of two-step PCR amplification was used: predenaturation at 95 °C for 30 s, PCR reaction at 95 °C for 5 s and at 60 °C for 30 s, a total of 40 cycles.

Table 2 The primers sequence

Primer name	Sequence
Gene amplification primer	F: TCACAACGCTGAAGGAAGTGATAGC
	R: GACCGGAGAAGCAGTGACGCAAAG
Gene fluorescence detection primer	F: CTAGGCATACACCGCTTCGT
	R: CCGGCGAGCCGAGATTATAG
Reference gene primer	F: ATGCTCCCAGGGCTGTATTC
	R: TACCCCTTTTAGACTGCGCC

Authors' contributions

YZ and ZZ were the executor of the experimental design and study. YZ, ZZ and YYP completed the data analysis and wrote the first draft of the paper. FSB and XW participated in the experimental design and analysis of experimental results. YZ was the proposer and leader of the project, directing experimental design, data analysis, paper writing and modification. All authors read and approved the final manuscript.

Acknowledgments

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