原著論文

Seasonal Changes of Citrus *Flowering Locus T* Gene Expression in Kumquat †1

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Summary

To elucidate the relation between flowering related genes and floral development in kumquat (genus *Fortunella*), morphological observation by microscope and transcription analysis were conducted. Flower bud formation was observed under the microscope after 4 June and stamens were observed after 18 June. The gene expression of citrus Flowering Locus T (*CiFT*) was increased from May to July in the leaves and from middle June to late July in the stem. The levels in the stems were lower than those in the leaves. In citrus *LEAFY* homologue (*CsLFY*), the gene expression began to increase during middle June in the stem and late July in the leaves, and peaked in late July or middle August, respectively. Our results showed that the *CiFT* and *CsLFY* expression in leaves and stems increased during floral induction and flower bud development in kumquat. These results suggest that the *CiFT* and *CsLFY* may be related with floral induction and/or flower bud development in kumquat.

Key words: floral induction, flowering, *Fortunella*, *FT*, *LEAFY*.

Introduction

Flowering is one of the most important events in plant life cycle. During the last two decades, molecular information of flowering was accumulated (Mouradov et al., 2002, Kobayashi and Weigel 2007; Farrona et al., 2008). Recent research reported that protein of the *Flowering Locus T (FT)* gene, its rice homologue, *Hd3a*, and tomato homologue, SFT, act as mobile flowering signals (Abe et al. 2005; Wigge et al. 2005; Lifschitz et al. 2006; Corbesier et al., 2007; Jaeger and Wigge, 2007; Lin et al. 2007; Mathieu et al., 2007; Tamaki et al., 2007; Notaguchi et al., 2008). Another flowering-related genes such as *APETALA (AP) 1* and *LEAFY (LFY)* has been reported as flower meristem identity genes, which regulate flower organ identity genes such as *AP1*, *AP2*, *AP3*, *PISTILLATA*, *AGAMOUS*, *SEPALLATA (SEP) 1*, *SEP2* and *SEP3* (Jack 2004; Robles and Pelaz., 2005). On the other hand, it has been suggested that *TERMINAL FLOWER 1 (TFL1)* is an important repressor of floral induction in *Arabidopsis* (Ohshima et al., 1997).

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In horticultural plants, the flowering connects with productivity and it is important to understand the flowering characteristics of the crops. Kumquat (genus *Fortunella*) is one of the economical fruits in *Rutaceae* and close relative to genus *Citrus* and *Poncirus*. Both *Fortunella* and *Citrus* are evergreen but *Poncirus* shows deciduousness. In spite of their relativeness, their seasonal flowering characteristics are largely different (Fig. 1). In satsuma mandarin (*C. unshiu*), floral induction occurred by low temperature during fall and winter but flower organ is not observed until January (Inoue, 1989, 1990; Iwasaki, 1959; Krajewski and Rabe, 1995). As the temperature rises in spring, flower organ development proceeds and blooms in May. In trifoliate orange (*P. trifoliata* L. Raf), floral induction occurs in early summer and subsequently, flower organ develops during summer (Spiegel-Roy and Goldschmidt, 1996). The flower organ developments stop during fall and winter. It starts again and blooms in spring. In kumquat, bud sprouting begins during April and May, one or two month later than those of satsuma mandarin and trifoliate orange. The development of shoots ceases late May and the flower organ development is observed in early summer. Subsequently, it blooms during summer. The bloom of kumquat usually occurs two or three times a season at approximately ten day-intervals (Yoshida et al., 2003).

Previously, we reported that seasonal expression of flowering genes in the stem of trifoliate orange and satsuma mandarin (Nishikawa et al., 2007, 2009). Out of the flowering genes, seasonal gene expression of citrus *FT (CiFT)* and citrus *LFY (CsLFY)* was enhanced during floral induction and/or flower organ development in both species although their seasonal periodicity of flowering is different. These results suggest that *CiFT* and *CsLFY* were profoundly associated with floral induction and/or flower organ development. In this study, seasonal gene expression patterns of flowering genes were characterized in kumquat, in which flowering season was different with other citrus relative species.

Materials and Methods

1. Plant materials

Adult kumquat 'Puchimaru' (*Fortunella crassifolia* Swingle ×*F. margarita* Swingle) trees was used for the material. The trees were grown in the field at Okitsu and Kuchinotsu citrus research stations of National Institute of Fruit Tree Science (NIFTS). The axillary buds were collected from May to June for the morphological observation by microscope. To investigate seasonal changes in mRNA levels, stems without leaves, which included internodes and nodes from the base to the apex, and leaves were collected and immediately frozen in liquid nitrogen.

2. Tissue fixation, embedding and sectioning

The collected buds were immediately fixed in FAA (1.8% formaldehyde, 5% acetic acid, 50% ethanol) or PFA (5% paraformaldehyde, 1% grutalaldehyde, 10mM Na-phosphate ｐH7.4, 100mM NaCl) over night. Fixed samples were dehydrated using ethanol: xylene series, and then embedded in Paraplast Plus (Sigma-Aldrich Co., St Louis, MO, USA). The embedded samples were stored at 4℃ until use. The embedded buds were sliced into 8-μm sections, and the sections were mounted on coated microscope slides (Matsunami Glass Ind., Kishiwada, Japan). Paraffin was removed by washing twice with xylene and mounted with Entellan new (Merk Chemical, Darmstadt, Germany). The sections were viewed through a phase contrast microscopy.

3. Total RNA extraction and real-time PCR

Extraction of total RNA and cDNA synthesis was carried out according to the methods of Nishikawa et al. (2007). For SYBR Green real-time PCR, primers for *CiFT*, *CsLFY*, *CsAP1* and citrus *TFL1 (CsTFL)*, were designed with the Primer Express software (Applied Biosystems) (Nishikawa et al., 2009). As an endogenous control, the EF1-α primers were used (Nishikawa et al., 2009). SYBR Green real-time PCR was performed with the Power SYBR Green Universal PCR Master Mix (Applied Biosystems) using an ABI PRISM 7000 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions. Each reaction contained 900 nM primers and 2.5 μl of tem-

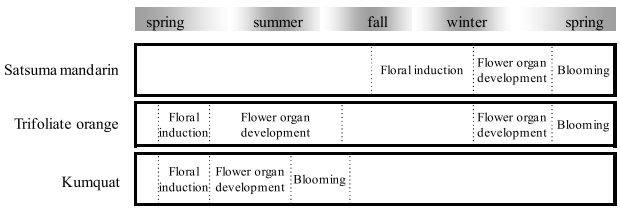

Fig. 1. Schematic diagram of seasonal flowering in Satsuma mandarin (*C. unshiu*), trifoliate orange (*P. trifoliata*) and kumquat (*Fortunella*).

plate cDNA. The thermal cycling conditions were 95℃ for 10 min followed by 40 cycles of 95℃ for 15 s and 60℃ for 60 s. The levels of gene expression were analyzed with ABI PRISM 7000 Sequence Detection System software (Applied Biosystems). Real-time quantitative PCR was performed in three replicates for each sample and data are presented as means ± SD (n = 3).

Results and Discussions

In kumquat, spring shoots were sprouted in the end of April and development of shoot length was stopped by June. In middle June, flower buds were observed with naked eyes and they bloomed in middle July. To investigate the beginning and progression of flower organ development, axillary buds were collected and observed under the microscopes from May to June. Microscopic examination showed that axillary buds had a narrow and smooth dome on 7 May and 22 May and the dome was broadened by 4 June (Fig. 2). At 11 June, the buds domed considerably and primordia of pistils and stamens were observed at 18 June.

The seasonal expression pattern of flowering genes was investigated to elucidate the association with the floral induction and flower development in kumquat. In kumquat leaves, the *CiFT* mRNA levels were increased rapidly from 22 May to 2 July, during initiation period of floral bud formation occurred (Fig. 3). The levels peaked in early July and then rapidly decreased by August. In the stem, transcripts of *CiFT* showed a peak in late July but the level at the peak was much lower than that in the leaves. After August, the *CiFT* gene expression remained at low levels in both tissues. In *CsLFY*, the gene expression increased after 11 June in the stems and after 23 July

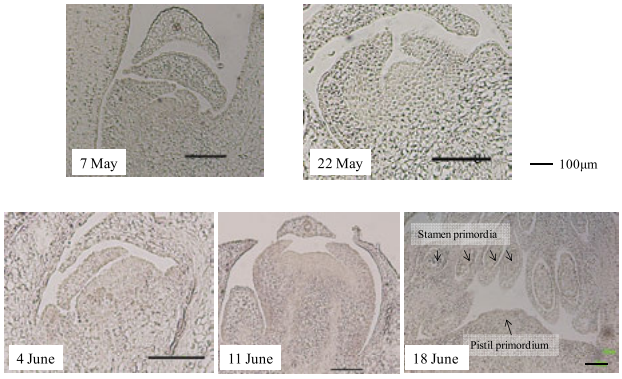

Fig. 2. Morphological changes of axillary buds of 'Puchimaru'. Axillary buds from spring shoots were fixed in FAA or PFA and embedded in Paraplast plus. Longitudinal sections (8- μ m thickness) of axillary buds were prepared. Arrow heads show the stamen or pistil primordia.

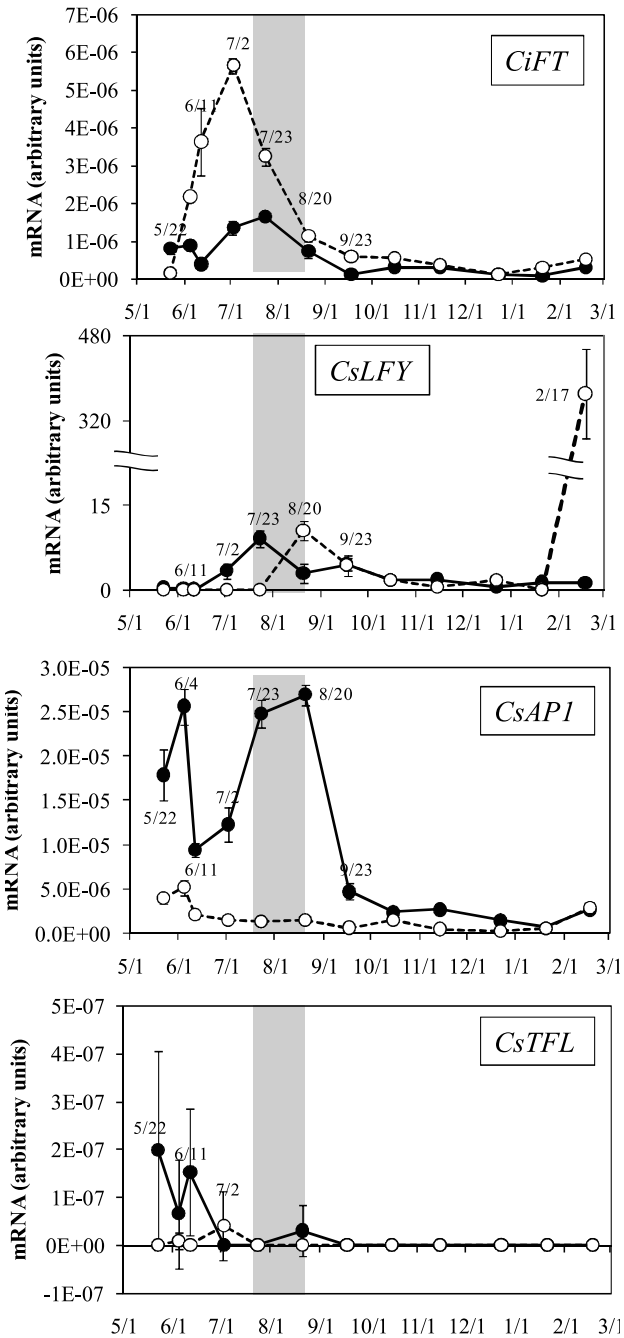

Fig. 3. Gene expression of *CiFT*, *CsLFY*, *CsAP1* and *CsTFL* of kumquat in stems (closed circle) and leaves (open circle) collected through the year. SYBR Green real-time PCR was performed with gene specific primers. Data are mean ± SD (n=3). Gray zone shows the blooming period.

in the leaves, and peaked at 23 July or 20 August, respectively. After the peak, the gene expression decreased and remained at low levels until January in both tissues. In the leaves, a considerable increase was observed in the *CsLFY* mRNA levels in February. The transcript amounts of *CsAP1* showed relatively high from May to August in kumquat stems. In the leaves, *CsAP1* mRNAs were detected at low levels through the experimental period. The mRNA abundance for *CsTFL* was detected during early summer but undetected after September in the stem of kumquat. In the leaves, *CsTFL* mRNA was rarely detected through the experimental period.

In our data, initiation of floral formation and increase in *CiFT* expression in the leaves were observed simultaneously during early June in kumquat. These results suggest that the *CiFT* expressed in the leaves may influence the seasonal floral induction in kumquat. In *Arabidopsis* and rice, a photoperiod signal is received in their leaves, in which *FT* and *Hd3a* mRNA are expressed, respectively. In perennial woody species, poplar, it has been reported that photoperiod effects poplar *FT* gene expression in the leaves and that transcript amounts of poplar *FT* in the leaves peaked in July during the floral initiation period (Böhlenius et al., 2006; Hsu et al., 2006). Our results in kumquat seem to agree with those of *Arabidopsis*, rice and poplar. In the stem, the mRNA levels of *CiFT* also increased during summer although the initiation of the increase and the time at the peak were later than those in the leaves. In the previous paper, we reported that initiation of floral formation occurred along with the *CiFT* expression in the stem of trifoliate orange (Nishikawa et al., 2009). These results suggest that *CiFT* expression both in the stem and leaves might correlate with floral induction in kumquat. In Satsuma mandarin, *CiFT* expression is induced by low temperature during fall and winter. Both the time of floral induction and of increase in the *CiFT* expression is largely different between Satsuma mandarin and kumquat. In kumquat, the *CiFT* expression seems to be regulated by factors other than low temperature since the expression increased under high temperature during summer. Therefore, these results suggest that floral induction and *CiFT* expression in kumquat may be regulated by the different factor from Satsuma mandarin.

In addition to *CiFT*, the *CsLFY* mRNA level increased during summer in the stem of kumquat. However, the timing of increase was later than that of the initiation of morphological changes in the axillary buds. In the microscopic observation, the first changes to flower organ development were observed at 4 June, during which the *CsLFY* expression in the stem remained at low level. The *CsLFY* expression in the stem was increased after 11 June, during which the stamen and pistil primordia was formed rapidly. In our previous paper, it has been suggested that *CsLFY* plays a role in flower bud development morphologically (Nishikawa et al., 2009). These results suggest that the *CsLFY* may correlate with morphological development of flower bud and less with the floral initiation in kumquat as those in other relative species.

The *CsAP1* mRNA level was high during spring and summer but low during fall and winter in the stem of kumquat. The pattern is similar to those in the stem of trifoliate orange, which was reported previously (Nishikawa et al., 2009). This result suggests that *CsAP1* may correlate with flower bud development both in kumquat and trifoliate orange during summer. Previously, we suggested the correspondence between the *CsAP1* expression and seasonal flowering differed between Satsuma mandarin and trifoliate orange. In Satsuma mandarin, the *CsAP1* expression was low during floral induction and flower bud development. These results suggest that the relation between *CsAP1* and seasonal flowering in kumquat is also different from that in Satsuma mandarin.

Changes in the *CsTFL* mRNA in the stem of kumquat showed a similar pattern with those of trifoliate orange and Satsuma mandarin (Figs.3; Nishikawa et al., 2009). The expression of *CsTFL* may be regulated by a same factor in those species. Low levels of *CsTFL* transcription is in good agreement with the role of *CsTFL* as a flowering repressor (Pillitteri et al., 2004).

In conclusion, our data showed that the *CiFT* mRNA levels in the leaves of kumquat increased from May to July and that morphological development of flower buds began to be observed in early June. The expression of *CsLFY* in the stems also increased from middle June to July, during which the pistil and stamen developed rapidly. These results suggest that *CiFT* and *CsLFY* may correlate with floral induction and/or flower bud development during early summer in kumquat. The flower blooming in kumquat occurs several times in a season. The molecular mechanism of this characteristic is still unclear from our

results. Further studies are required to understand flowering behaviors fully in kumquat.

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キンカンにおける*CiFT*遺伝子発現の季節変化

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摘　要

キンカンの花芽の発達と花成関連遺伝子との関連を明らかにするために、顕微鏡による形態観察と発現解析を行った。形態的な花芽形成は顕微鏡下で6月4日以降に観察され、雄ずいは6月18日以降に観察された。カンキツ*Flowering Locus T*ホモログ（*CiFT*）の発現は、葉で5月から7月に、茎組織では6月中旬から7月下旬に増大した。茎での発現量は葉のものより低かった。カンキツ*LEAFY*ホモログ（*CsLFY*）では、発現が茎で6月初旬に、葉では7月下旬に増大し始め、それぞれ7月下旬、8月中旬にピークに達した。我々の結果はキンカンの花成および花芽の発達の時期に茎および葉における*CiFT*と*CsLFY*の発現が増大することを示した。これらの結果は*CiFT*と*CsLFY*がキンカンの花成あるいは花芽の発達に関与していることを示唆している。