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原著論文

Leaf Removal Suppresses Citrus *FLOWERING LOCUS T* Expression in Satsuma Mandarin

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Summary

Citrus trees are evergreen but sometimes defoliate when exposed to excess winter cold, salty wind injury, or serious drought. Defoliation often decreases flower number in the following spring. To understand the underlying molecular mechanism, we investigated the effect of defoliation on a flowering-related gene, citrus *FLOWERING LOCUS T (CiFT)*. To determine the effect of defoliation period on *CiFT* expression, potted satsuma mandarin trees (*Citrus unshiu* Marc.) were completely defoliated at different time while growing at 15℃, the floral-inductive temperature. After 2.5 months at 15℃, *CiFT* expression was higher in the stems of trees that retained their leaves for longer periods. These results indicated that early defoliation suppressed *CiFT* expression in the stem. To determine how leaf number affected *CiFT* expression, potted trees were defoliated to different degrees and grown at 15℃ for one month. *CiFT* expression in the stem was strongly and positively correlated with leaf number, indicating that a decrease in leaf number suppressed *CiFT* expression in the stem. In both experiments, floral induction at the end of 15℃ treatment was estimated by forcing the trees to sprout at 25℃. Floral induction was suppressed by early or extensive defoliation, corresponding with the changes in *CiFT* expression.

Key words: *CiFT*, *Citrus unshiu* Marc., defoliation, floral induction, *FT*.

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Introduction

Citrus trees are evergreen but sometimes defoliate when exposed to excessive winter cold, or salty wind injury. Recently, mandarin trees have been grown under serious water-deficient conditions to produce high-sugar fruits. Such drought stress often causes defoliation. In these cases, defoliated trees bear few flowers and fruits in the following year, disrupting the balance between vegetative and reproductive growth. Once this balance is disrupted, the annual number of flowers often becomes unstable, and the trees alternate between rich and poor crops; this condition is known as alternate bearing. Because alternate bearing interrupts a steady fruit supply, its prevention is important for citrus fruit production. Thus, understanding the mechanism by which defoliation can suppress flowering and trigger alternate bearing is important.

In some herbaceous plants, leaves have been reported to play a crucial role in floral induction. In Arabidopsis, a long day is one of the main signals promoting flowering. This signal is detected by leaves, which then express the flowering-promoting gene *FLOWERING LOCUS T (FT)* (Takada and Goto, 2003). The *FT* protein is transferred from leaves to shoot apices, where it regulates flower bud differentiation (Corbesier et al., 2007). Thus, mRNA for *FT* is produced in leaves, while the protein itself is a mobile flowering signal. Citrus *FT* homologues (*CiFT*) promote flowering in citrus (Endo et al., 2005). In satsuma mandarin (*Citrus unshiu* Marc.), *CiFT* expresses in the stems, leaves, and fruits (Nishikawa et al., 2007). When the tree is exposed to floral-inductive temperatures (15°C), *CiFT* expression increases in both stems and leaves. However, the mRNA levels in stems appear to be more strongly correlated with floral induction than those in leaves (Nishikawa et al., 2007). Previously, we reported that *CiFT* mRNA levels in stem and leaf tissues during fall and winter were highly correlated with flower number the following spring in satsuma mandarin trees that bore different amounts of fruits (Nishikawa et al., 2012). However, the correlation in leaf tissues was weaker than in stem tissues. Those results implied that endogenous *CiFT* promoted seasonal floral induction and that *CiFT* mRNA levels in the stem played a more important role in floral induction than those in leaves.

In this study, the effect of artificially removing leaves on *CiFT* expression levels in the stem of satsuma mandarin was investigated. *CiFT* expression correlated with the timing of leaf elimination and the number of leaves on the tree. On the basis of these results, we discuss the molecular mechanism by which defoliation suppresses flowering.

Materials and Methods

Plant materials

To investigate the effect of leaf removal under flowering-inductive conditions, 1-year-old potted 'Okitsu-wase' satsuma mandarin trees grafted onto trifoliate orange (*Poncirus trifoliata* (L.) Raf.) trees were purchased from a local market in early spring. The potted plants were grown outdoors until July, by which time spring flushes had sprouted and completed hardening. All of the plants were transferred in early July to rooms held at 15°C and kept there for 1 or 2.5 months, depending on the experiment. Floral induction at the end of the 15°C treatment was estimated as described by Inoue (1990). At the end of the 15°C treatment, all leaves were defoliated and transferred to a 25°C room to force the trees to sprout. Then, flower buds were counted as a measure of floral induction. For RNA extraction, a few stems of spring vegetative shoots were collected from each plant at the end of the 15°C treatment. These samples were immediately frozen in liquid nitrogen and stored at −80°C until use. Throughout the experimental period, the trees were watered regularly, and the light conditions were the same as outdoors. In this study, two experiments were conducted, as described below.

Experiment 1: To investigate how the timing of defoliation affected floral induction, all leaves were removed from each of three satsuma mandarin trees after either 0, 0.5, 1, 1.5 or 2 months of the 15°C treatment ; another three trees were not defoliated for 2.5 months of the 15°C treatment (control). After 2.5 months of treatment, all trees were transferred to a 25°C room and control trees were completely defoliated to force sprouting for estimation of floral induction.

Experiment 2: To investigate how the degree of leaf removal affected floral induction, each of five trees were set to retain 0, 50, 75, or 100% of their original leaf numbers; the trees were then placed in a 15°C room for one month. All leaves of all the plants were removed at the end of the month, when the trees were transferred to 25°C and

floral induction was measured, and *CiFT* expression was analyzed (see below).

Total RNA extraction and real-time PCR

For real-time reverse transcription (RT)-PCR analysis, total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and cleaned by on-column DNase digestion. The RT reactions were performed with 0.4 μg of purified total RNA and a random hexamer at 37°C for 2 h by using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA).

TaqMan minor grove binder (MGB) probes and sets of primers for total *CiFT* were designed using Primer Express software (Applied Biosystems) (Nishikawa et al., 2007). For an endogenous control, the TaqMan Ribosomal RNA Control Reagents VIC Probe (Applied Biosystems) was used. TaqMan real-time PCR was carried out with the TaqMan Universal PCR Master Mix (Applied Biosystems) using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. Each reaction contained 900 nM primers, 250 nM TaqMan MGB Probe, and 2.5 μL of template cDNA. The thermal cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The levels of gene expression were analyzed with ABI PRISM 7000 Sequence Detection System Software (Applied Biosystems) and normalized with the results of 18S ribosomal RNA. Real-time quantitative RT-PCR was performed in triplicate for each sample and data were shown as the mean of the logarithmic value ± SE (n=3).

Results

In this study, we conducted two experiments on the timing (experiment 1) and extent (experiment 2) of leaf-removal in satsuma mandarin trees. The effects of these treatments on floral induction and *CiFT* expression were investigated.

Experiment 1: Exposure to 15°C for 2.5 months is generally sufficient for floral induction in satsuma mandarin trees. Non-defoliated control trees bore around 3.5 flower buds per node, indicating that floral induction was promoted fully by the temperature treatment. Trees that were defoliated at 0 or 0.5 month had significantly fewer flower buds than the control ($P<0.05$, Fig. 1). In those defoliated at 1, 1.5, or 2 months, floral induction was moderate. Thus, the average number of flower buds decreased as the period without leaves increased, indicating that prolonged leaflessness at 15°C suppressed floral induction. Transcript levels of *CiFT* paralleled the number of flower buds. In the trees defoliated at 0 or 0.5 months, *CiFT* mRNA levels were the lowest. The *CiFT* mRNA level of non-defoliated trees was about 100 times that of those defoliated at 0 months. The *CiFT* mRNA levels tended to increase with the length of time the trees retained their leaves. These data indicated that longer periods of leaflessness suppress *CiFT* transcription.

Experiment 2: Generally, flowering of citrus is induced gradually by exposure to 15°C. In this experiment, trees were exposed to 15°C for only one month, which is sufficient only to begin floral induction. In the control plants (100% of leaves remaining), there were about 0.25 flower buds per node after transfer to a 25°C room. This number was substantially different from that of the non-defoliated control trees in experiment 1, which experienced 15°C for

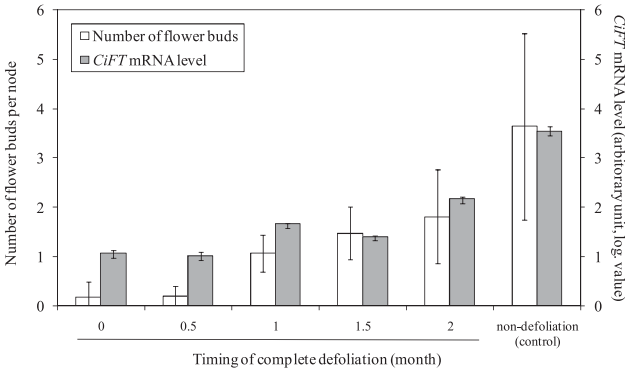

Fig . 1. Floral induction (white bars) and *CiFT* mRNA levels (gray bars) in satsuma mandarin trees defoliated of all leaves at different times. Potted trees were placed in a 15°C room for 2.5 months, and which all of the leaves were removed after either 0, 0.5, 1, 1.5, or 2 months or not at all (control). There were three trees per treatment. After 2.5 months, plants were transferred to a 25°C room, and floral induction was estimated as the number of flower buds sprouted. Data are means ± SE (n=3). Expression of *CiFT* in the stems was analyzed by TaqMan real-time quantitative RT-PCR with gene-specific probe and primers. Gene expression data are given as the mean (logarithmic value) ± SE (n=3).

2.5 months. This means that floral induction started at around one month and proceeded considerably between 1 and 2.5 months under these conditions in these experiments. In this experiment, although the trees were in the early stages of floral induction, the amount of leaves affected both floral induction and *CiFT* expression. Trees that retained 100% of their leaves had the highest average of number of flower buds after transfer to 25°C, followed in order by those that retained 75% and 50% of their leaves; no buds were formed on trees that had been fully defoliated (Fig. 2). Thus, leaf quantity positively affected floral induction, which was completely suppressed by too few leaves. The *CiFT* mRNA levels paralleled floral induction; mRNA levels were highest in the 75% and 100% treatments, moderate in trees with 50% of their leaves, and lowest in fully-defoliated trees. The trees retaining 100% of their leaves had about 100 times the *CiFT* mRNA levels as those that were of completely defoliated. Thus, leaf quantity regulates *CiFT* transcription in the stem, and *CiFT* expression is suppressed by a decrease in leaf number.

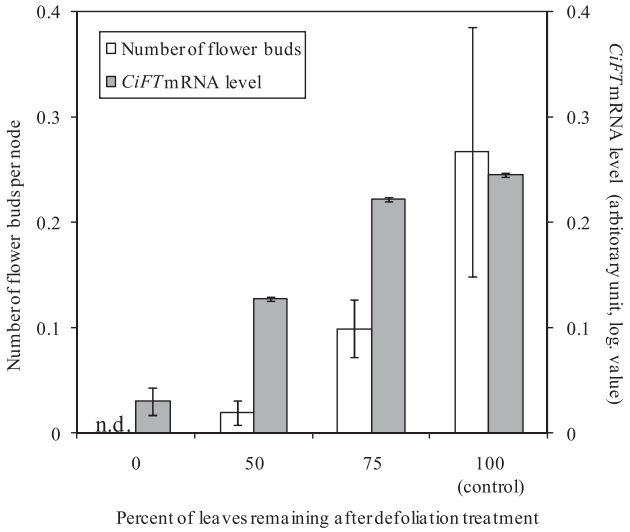

Fig. 2. Floral induction (white bars) and *CiFT* mRNA levels (gray bars) in satsuma mandarin trees after defoliation followed by growth at 15°C for one month. Plants were defoliated to 0, 50, 75, or 100% of the original leaf number. Floral induction was estimated by the number of flower buds per node after forced sprouting at 25°C. Data are means ± SE (n=5). n.d., not detected. Expression of *CiFT* in stems was analyzed by TaqMan real-time quantitative RT-PCR with gene-specific probe and primers. Gene expression data are given as the mean (logarithmic value) ± SE (n=3).

Discussion

In this study, we investigated the effects of leaf removal on floral induction and *CiFT* expression. Our results indicated that the timing and amount of leaf removal affected both floral induction and *CiFT* expression. Both early and more extensive leaf removal suppressed floral induction and *CiFT* expression. These results suggested that leaf persistence was important for cold-induced flowering and *CiFT* expression.

In our experiments, *CiFT* expression levels were closely correlated with floral induction, implying that floral induction was suppressed by leaf removal via the suppression of *CiFT* expression. As described above, *CiFT* promotes flowering, and changes in endogenous *CiFT* expression are consistent with floral induction caused by low temperature (Endo et al., 2005; Nishikawa et al., 2007). Our previous study showed that fruiting suppressed *CiFT* expression during fall and winter and flower number in the following spring (Nishikawa et al., 2012).

In addition to *CiFT*, citrus homologues of many flowering-promoting genes, such as *APETALA1 (AP1)*, *LEAFY (LFY)*, *SEPALLATA (SEP) 1*, *SEP3*, and *FRUITFULL* have been identified so far (Pillitteri et al., 2004a, 2004b; Nishikawa et al., 2010). However, the mRNAs of those genes did not accumulate during the floral induction period under natural or artificial conditions (Pillitteri et al., 2004b; Nishikawa et al., 2007, 2009). Expression of *AP1*, *LFY*, *SEP1*, and *SEP3* increased during the stage of morphological flower bud development (Pillitteri et al., 2004b; Nishikawa et al., 2009). These data indicate that those genes may not trigger seasonal floral induction in citrus. A homologue of *TERMINAL FLOWER 1* in citrus (*CsTFL*) has been reported to suppress flowering, and *CsTFL* mRNA was detected at high levels in juvenile citrus stems (Pillitteri et al., 2004b). In adult plants, *CsTFL* mRNA was rarely detected except stems collected in June (Nishikawa et al., 2007). Because citrus flowering is induced by decreased temperatures during fall and winter, *CsTFL* may not be a direct trigger of floral induction under natural conditions. However, the possibility that defoliation suppresses floral induction via induction of *CsTFL* expression cannot be neglected. At the present time, only *CiFT*

has been recognized as a candidate gene triggering floral induction in seasonal periodicity of citrus. Therefore, we focused on *CiFT* in this study. Our results support the idea that endogenous *CiFT* expression may be a major factor regulating floral induction.

Based on our results, we hypothesize that floral induction is dependent on the length of exposure to 15°C when leaves are attached. For example, trees defoliated at 1.5 months had about 1.5 flower buds per node, more than the completely-defoliated trees in experiment 2, which were not exposed to 15°C while their leaves were attached. In both treatments, trees were exposed at 15°C for one month after leaf removal. These results suggest that floral induction proceeded considerably during the 1.5 months of 15°C incubation before defoliation and that flowering potential persisted during the defoliated condition in experiment 1. Our results also suggested that defoliation does not completely suppress floral induction. In the trees defoliated at 1 month in experiment 1, floral induction was greater than in non-defoliated trees in experiment 2. In both treatments, trees experienced 15°C for 1 month with leaves attached. Because the trees defoliated at 1 month in experiment 1 remained exposed to 15°C for 1.5 months after defoliation, the different number of flower buds may have resulted from the length of exposure to 15°C after leaf removal. Thus, we concluded that floral induction during exposure to 15°C increased greatly when leaves were attached and only slightly when trees were defoliated.

Our study indicated that defoliation suppressed both floral induction and *CiFT* expression. However, how defoliation caused these effects is unclear. Defoliation causes many physiological changes related to transport of substances, transpiration, and wounding. In defoliated trees, these changes may correlate with the suppression of floral induction and *CiFT* expression. Further research will be needed to understand how defoliation suppresses *CiFT* expression in the stem.

References

1) Corbesier, L., C. Vincent, S. Jang, F. Fornara, Q. Fan, I. Searle, A. Giakountis, S. Farrona, L. Gissot, C. Turnbull and G. Coupland. (2007). FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. Science 316: 1030–1033.

2) Endo, T., T. Shimada, H. Fujii, Y. Kobayashi, T. Araki and M. Omura. 2005. Ectopic expression of an *FT* homolog from Citrus confers an early flowering phenotype on trifoliate orange (*Poncirus trifoliata* L. Raf.). Transgenic Res. 14: 703–712.

3) Inoue, H. 1990. Effects of temperature on bud dormancy and flower bud differentiation in satsuma mandarin. J. Japan Soc. Hort. Sci. 58: 919–926. (in Japanese with an English summary).

4) Nishikawa, F., T. Endo, T. Shimada, H. Fujii, T. Shimizu, M. Omura and Y. Ikoma. 2007. Increased *CiFT* abundance in the stem correlates with floral induction by low temperature in satsuma mandarin (*Citrus unshiu* Marc.). J. Exp. Bot. 58: 3915–3927.

5) Nishikawa, F., T. Endo, T. Shimada, H. Fujii, T. Shimizu and M. Omura. 2009. Differences in seasonal expression of flowering genes between deciduous trifoliate orange and evergreen satsuma mandarin. Tree Physiol. 29: 921–926.

6) Nishikawa, F., T. Endo, T. Shimada, H. Fujii, T. Shimizu, Y. Kobayashi, T. Araki and M. Omura. 2010. Transcriptional changes in *CiFT*-introduced transgenic trifoliate orange (*Poncirus trifoliata* L. Raf.). Tree Physiol. 30:431–439.

7) Nishikawa, F., M. Iwasaki, H. Fukamachi, K. Nonaka, A. Imai, F. Takishita, T. Yano and T. Endo. 2012. Fruit bearing suppresses citrus *FLOWERING LOCUS T* expression in vegetative shoots of satsuma mandarin (*Citrus unshiu* Marc.). J. Japan Soc. Hort. Sci. 81: 48–53.

8) Pillitteri LJ., CJ. Lovatt and LL. Walling. 2004a. Isolation and characterization of *LEAFY* and *APETALA1* homologues from *Citrus sinensis* (L.) Osbeck 'Washington'. J. Amer. Soc. Hort. Sci. 129: 846–856.

9) Pillitteri LJ., CJ. Lovatt and LL. Walling. 2004b. Isolation and characterization of a *TERMINAL FLOWER* homolog and its correlation with juvenility in citrus. Plant Physiol. 135: 1540–1551.

10) Takada, S. and K. Goto. 2003. *TERMINAL FLOWER2*, an Arabidopsis homolog of *HETE-*

ROCHROMATIN PROTEIN1, counteracts the activation of *FLOWERING LOCUS T by CONSTANS* in the vascular tissues of leaves to regulate flowering time. Plant Cell 15: 2856–2865.

ウンシュウミカンにおいて摘葉はカンキツ *FLOWERING LOCUS T* の発現を抑制する

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摘　要

常緑性であるカンキツ樹は，寒害や潮風害，乾燥にさらされ時々落葉する．落葉はしばしば翌春の花数を減少させる．落葉による花成抑制の分子機構を明らかにするために，我々は花成制御遺伝子*CiFT*の発現に及ぼす落葉の影響を調査した．*CiFT*の発現における着葉期間の影響を調査した実験では，花成誘導温度である15℃に置かれた鉢植えのウンシュウミカン樹から異なる時期にすべての葉が切除された．15℃に移してから2.5ヶ月目において，*CiFT*はより長期間着葉していた樹の茎組織で高く発現する傾向にあった．このことは早期の摘葉が茎の*CiFT*の発現を抑制することを示している．*CiFT*の発現における葉数の影響を調査した実験では，鉢植え樹が異なる葉数を持つように調整し，15℃に置いた．15℃に置いてから1ヶ月目に，茎における*CiFT*の発現は葉数と正の高い相関を示した．両実験において，15℃処理終了時点での花成を25℃において強制的に発芽させた後に観察される花芽数によって評価した．花成は早期の摘葉および大量の摘葉によって抑制され，*CiFT*の発現変動と一致していた．