Fruit Bearing Suppresses Citrus *FLOWERING LOCUS T* Expression in Vegetative Shoots of Satsuma Mandarin (*Citrus unshiu* Marc.)

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Citrus trees alternate between rich and poor crops and are known to be alternate-bearing crops. Alternate bearing results from suppression of flowering due to bearing of fruits. To understand the molecular mechanism how fruit bearing affects flowering, we investigated the relationship between fruits and a flowering-related gene, citrus *FLOWERING LOCUS T* (*CiFT*). On trees with different amounts of fruits, the fruit weight/leaf area ratio at harvest was negatively and highly correlated with *CiFT* expression in the vegetative shoots during fall and winter, which is the period of floral induction. In addition, *CiFT* expression levels during fall and winter were positively and highly correlated with the flower number the following spring. These results indicate that fruit growth suppresses *CiFT* expression and decreases the flower number the next spring. In another experiment conducted to determine the effect of the period of fruit bearing on *CiFT* expression, trees having 3 primary scaffold branches were analyzed. From each branch in 1 tree, all the flowers or fruits were harvested at different times. In November, *CiFT* was expressed at different levels in each branch, with a tendency to be low in the stem of vegetative shoots from branches that bore fruits for longer periods. This result indicates that a long fruit-bearing period suppresses *CiFT* expression in vegetative shoots. *CiFT* expression was detected at much higher levels in fruit-bearing shoots than in vegetative shoots in September. In January, the high levels of *CiFT* expression in bearing shoots decreased to levels lower than those found in vegetative shoots. Thus, in fruit-bearing shoots, the *CiFT* expression of an unknown relationship to floral induction is observed during the period before floral induction and the seasonal change of *CiFT* expression in fruit-bearing shoots is different from that in vegetative shoots.

Key Words: alternate bearing, *CiFT*, flowering, fruit, *FT*.

Introduction

In many fruit trees, the tree condition of the previous year influences the fruit production of the tree in the following year. In citrus trees, the amount of fruit and the time of harvest in the previous year influence the number of flowers growing in the following season: excess fruit bearing and late harvest reduce the flower numbers and production. Bearing shoots (shoots with flowers) produced in the previous year tend to produce only vegetative shoots (spring shoots without flowers) the following spring, whereas vegetative shoots produced in the previous year tend to produce flowers in the following year. Therefore, once the balance between the number of bearing and vegetative shoots is disrupted, the annual flower number becomes unstable, and the trees alternate between rich and poor crops; this is known as alternate bearing. Since this phenomenon interrupts a steady fruit supply, prevention of alternate bearing is important for the citrus industry. To control flower numbers, it is essential to understand the relationship between flowering and fruit bearing. Many studies have investigated the relationship between flowering and carbohydrates during alternate bearing. In a non-fruiting “off” year, trees accumulate and store large amounts of carbohydrates in their roots, whereas in a heavy-fruiting “on” year, the carbohydrate accumulation is less (Li et al., 2003). Yahata et al. (2006) reported that the time of fruit harvest affects starch accumulation in the shoots and that starch accumulation seems to be parallel with floral induction; however, the molecular mechanisms of the suppression of flowering due to fruit bearing or carbohydrate depletion are not understood.
During the past two decades, various studies have been conducted on flowering and many flowering-related genes have been identified in *Arabidopsis* (Komeda, 2004). Recently, the proteins of the Flowering Locus T (*FT*) gene and its rice homologue, *Hd3a*, were reported to act as mobile flowering signals, and these proteins have received considerable attention (Corbesier et al., 2007; Tamaki et al., 2007). It has been suggested that citrus *FT* homologues (*CiFT*) are key genes promoting flowering; in trifoliate orange (*Poncirus trifoliata* L. Raf.), a close relative of the genus *Citrus*, constitutive expression of *CiFT* resulted in extremely early flowering (Endo et al., 2005). Moreover, in adult satsuma mandarin (*C. unshiu* Marc.), endogenous expression of *CiFT* increased during fall and winter, concurrent with the seasonal floral induction (Nishikawa et al., 2007, 2009). Thus, *CiFT* expression can correlate with annual flowering in addition to the phase transition from juveniles to adults.

In this study, changes in *CiFT* expression due to fruit bearing were investigated. The *CiFT* expression during fall and winter showed a relationship with the leaf-to-fruit ratio and the timing of harvest. On the basis of these results, we discuss the molecular mechanisms of flowering suppression due to fruit bearing.

**Materials and Methods**

**Plant materials**

To investigate the relationship between the *CiFT* expression and the fruit weight per leaf area, 18-year-old ‘Aoshima’ satsuma mandarin trees grafted on trifoliate orange (*Poncirus trifoliata* L. Raf.) rootstock were grown in a field at the NARO Institute of Fruit Tree Science (NIFTS), Kuchinotsu (Minami-shimabara, Nagasaki, Japan), and 13 and 11 trees were used for transcriptional analyses of the stems and leaves, respectively. These trees had varying flower numbers in the spring of the experiment year because they had been harvested at different times in the previous year. From each tree, approximately 5 vegetative shoots were picked during the period of floral induction (November, December, and January). The vegetative shoots were divided into stems and leaves. Each tissue from about 5 vegetative shoots was mixed and then frozen. In December, all the fruits were harvested and weighed for each tree. After harvest, canopy volumes were measured by a scale for each tree. Leaf area density was also measured with a plant canopy analyzer (LAI2000, Li-Cor, Lincoln, USA), and the leaf area was calculated according to the method of Iwaya et al. (2005).

To investigate the effect of the timing of fruit picking, extremely early, early, and late-maturing satsuma mandarin (9-year-old ‘Hinosayaka’, 19-year-old ‘Haraguchi’, and 17-year-old ‘Aoshima’, respectively) grafted on trifoliate orange rootstock were grown in a field at NIFTS, Kuchinotsu, and one tree from each cultivar was used. Three primary scaffold branches were used from each tree. From one of these branches, all flowers were picked in May; from a second branch, all fruits were picked in August in extremely early and early-maturing type cultivars and in September in late-maturing type cultivars; and from the third branch, all the fruits were harvested in November. In November, the stems from approximately 5 vegetative shoots were harvested from each branch for RNA extraction.

To investigate *CiFT* expression in fruit-bearing shoots, we used ‘Aoshima’ trees grafted on trifoliate orange rootstock at NIFTS, Okitsu (Shizuoka, Japan); these were subjected to customary fruit thinning in August and the fruits were harvested in early December. From September to March, stems were collected every other month from fruit-bearing and vegetative shoots.

All samples for RNA extraction were immediately frozen in liquid nitrogen and stored at −80°C until use. The following spring, about 10 shoots were selected at random from each tree in the experiment for the fruit/leaf ratio, each branch for the timing of fruit picking, or each shoot for bearing shoots. For each shoot, the numbers of flowers, nodes or sprouting nodes were counted and the number of flowers per node or sprouting node was calculated.

**Total RNA extraction and real-time quantitative PCR**

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

A TaqMan MGB probe and sets of primers for total *CiFT* were designed using Primer Express software (Applied Biosystems) (Nishikawa et al., 2007). These probe and primers detect mRNAs for *CiFT1*, *CiFT2*, and *CiFT3* non-differentially. 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 system (Applied Biosystems) according to the manufacturer’s instructions. Each reaction contained 900 nM primers, 250 nM TaqMan MGB Probe, and 2.5 μL template cDNA. The thermal cycling conditions were 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. Gene expression levels were analyzed with the ABI PRISM 7000 Sequence Detection System Software (Applied Biosystems) and normalized to the results for 18S ribosomal RNA. Real-time quantitative RT-PCR was performed in 3 replicates for each sample, and data are shown as the mean of the logarithmic value ± SE (n = 3).
Results

Fruit weight per leaf area

To investigate the relationship between CiFT expression and fruit amounts, we analyzed mRNA levels of total CiFT in vegetative shoots from approximately 12 ‘Aoshima’ trees bearing different numbers of fruits. From November to January, the period of floral induction, logarithm values of the mRNA levels from both stems and leaves showed a negative correlation with the fruit weight per leaf area (Fig. 1, Table 1). In the stems, the mRNA levels in all trees increased from November to December and then decreased in January. On the other hand, in the leaves from almost all trees, no distinctive changes were observed in the mRNA levels from November to January. A correlation between the mRNA levels and the fruit weight per leaf area showed high coefficients from November to January (Table 1), with averages of −0.82 in the stems and −0.77 in the leaves. The CiFT mRNA levels showed a positive correlation with flower number per sprouting node, with coefficient averages of 0.85 in the stems and 0.71 in the leaves (Table 1). In this experiment, the correlation coefficient between fruit weight per leaf area and flower numbers per sprouting node was −0.62. Thus, the CiFT mRNA levels during fall and winter correlated closely with the leaf-to-fruit ratio at harvest and with the flower number in the following spring.

Timing of fruit picking

To investigate the effect of the timing of fruit picking, 3 cultivars of satsuma mandarin, namely, extremely early, early, and late-maturing types, were used. Flowers or fruits were picked in different seasons for 3 primary scaffold branches of 1 tree: all flowers were picked from 1 of the 3 branches in May, all fruits were picked from the second branch in August or September, and all fruits were picked from the third branch in November. All 3 cultivars showed similar trends of changes in CiFT expression (Fig. 2A). The CiFT mRNA levels in November were highest in the stems from vegetative shoots of the flower-picked branches in May and lowest in those that kept fruits until November. In branches picked in August or September, the CiFT expression levels were between those picked in May or November. These results show that the timing of fruit picking influences the CiFT mRNA levels in November. Of the 3 cultivars, the late-maturing type showed the highest mRNA levels of CiFT in the flower-picked branches, and the lowest levels were found in the late-maturing branches with fruit picked in November.

In each cultivar, the flower number per node in the following spring varied among the 3 branches. The trend was similar to those of CiFT mRNA levels in all 3 cultivars. The flower number was larger in the vegetative shoots of the flower-picked branches in May and smaller in those retaining fruits until November (Fig. 2B). These

Table 1. Correlation coefficient between the total CiFT mRNA levels and the amount of fruit or flower number (n = 13 for stems, n = 11 for leaves).

<table>
<thead>
<tr>
<th>Total CiFT mRNA levels</th>
<th>Fruit weight per leaf area (kg·m⁻²)</th>
<th>Flower number per sprouting node</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>stem</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nov.</td>
<td>−0.857 **</td>
<td>0.843 **</td>
</tr>
<tr>
<td>Dec.</td>
<td>−0.851 **</td>
<td>0.841 **</td>
</tr>
<tr>
<td>Jan.</td>
<td>−0.755 **</td>
<td>0.869 **</td>
</tr>
<tr>
<td><strong>leaf</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nov.</td>
<td>−0.664 *</td>
<td>0.742 **</td>
</tr>
<tr>
<td>Dec.</td>
<td>−0.863 **</td>
<td>0.745 **</td>
</tr>
<tr>
<td>Jan.</td>
<td>−0.791 **</td>
<td>0.643 *</td>
</tr>
</tbody>
</table>

* and ** indicate significance at 5% and 1% levels, respectively.
results indicate that flowering is suppressed by a long period of fruit bearing. Of the 3 cultivars, the highest flower number per node was observed in the flower-picked branches for the early-maturing type of satsuma mandarin, and lowest for the branches that retained fruits until November in the late-maturing type.

CiFT expression in bearing shoots

To compare CiFT expression in fruit-bearing shoots with that in vegetative shoots, RNA was extracted from bearing and vegetative shoots from September to March. In vegetative shoots, the CiFT expression increased from September to January and then decreased in March (Fig. 3). In fruit-bearing shoots, CiFT mRNA was detected at an extremely high level in September, the period before floral induction (Fig. 3). In November, the period of floral induction, CiFT transcription in the bearing stem was reduced to a level almost the same as that in vegetative shoots. After November, CiFT mRNA levels in bearing shoots were maintained at a decreased level, and lower levels than in vegetative shoots were detected in January. In March, the levels in bearing shoots increased slightly but were still lower than in vegetative shoots. Thus, seasonal changes in CiFT expression differed widely between vegetative and fruit-bearing shoots. The following spring, flowers were counted for each shoot, and the flower numbers per node were determined to be 0.54 and 0.00 in vegetative and fruit-bearing shoots, respectively.

Discussion

Fruit bearing and CiFT expression

In citrus, it is well known that too much fruit bearing in the previous year suppresses flower number and fruit production the following year. In this study, we show that the fruit weight per leaf area is negatively correlated with CiFT mRNA levels in vegetative shoots during fall and winter (Fig. 1). This result indicates that CiFT transcription in vegetative shoots could be suppressed by fruit bearing. Since CiFT has a role in floral induction, it is thought that the suppression of CiFT expression influences floral induction, which thereby influences flower number the following spring. Therefore, our results suggest that fruit bearing in the previous year suppresses flower number the following spring by suppressing CiFT transcription during fall and winter.

In addition to fruit amount per leaf area, the timing of fruit picking also affects CiFT mRNA levels in vegetative shoots (Fig. 2A). This means that the period of fruit bearing can regulate CiFT transcription. It has been reported that the flower number was reduced in trees that were harvested late in the previous year (Goldschmidt et al., 1985). In this experiment, the flower number per node was lower in the vegetative shoots of branches that bore fruits for longer periods (Fig. 2B). These results suggest that suppressing CiFT transcription by late fruit picking can cause a decrease of flower numbers. This experiment also showed that CiFT mRNA levels of vegetative shoots varied even in one tree, because the treatment was carried out for each branch of each tree. In vegetative shoots near fruits (e.g., those
in branches retaining fruits until November), CiFT expression was strongly suppressed by fruits, while the effects of fruits were weak in vegetative shoots far from fruits (e.g., those from flower-picked branches). Therefore, it is likely that the effect of fruit bearing on CiFT expression in vegetative shoots is limited to their vicinity to the fruit-bearing portion of the branch. Taken together, our results show that the conditions of fruit bearing, including the leaf-to-fruit ratio and the timing of fruit picking, can influence the CiFT transcription of the stems of vegetative shoots. In citrus, it has been reported that fruit bearing causes some physiological changes. Starch depletion in shoots is one of the changes by fruit bearing (Yahata et al., 2006). In addition, it has been reported that endogenous GA levels are higher in fruit-bearing shoots than in vegetative shoots in November (Koshita et al., 1999; Takagi et al., 1989). Treatment with exogenous GA in November has been shown to inhibit flower bud formation in citrus (Hirose, 1968; Inoue, 1990a); therefore, it is thought that GA or starch might correlate with fruit bearing and flowering and that fruit bearing might suppress CiFT expression through those substances.

Citrus flowering is induced by temperatures of approximately 15°C during the fall and winter (Inoue, 1990b). In fruit-bearing shoots, extremely high levels of CiFT mRNA were detected in September (Fig. 3). Since citrus trees have not yet been exposed to temperatures under 25°C in September, high CiFT transcription seems to have nothing to do with exposure to flower-inductive temperatures. In some plant species, other roles of FT have been reported besides flowering. In potato, FT works on tuberization (Rodriguez-Falcon et al., 2006), and the FT homologue in poplar plays a role in dormancy (Böhlenius et al., 2006). Thus, in the stem of fruiting shoots, CiFT might have an unknown developmental role other than floral induction. In the previous study, we reported that 3 CiFT homologues (CiFT1, CiFT2, and CiFT3) were expressed in satsuma mandarin and that high mRNA levels for CiFT1 and 2 were detected in fruits (Nishikawa et al., 2007). Their transcript levels in the stem and leaves of vegetative shoots did not correlate with floral induction. In this study, we used sets of probe and primers for total CiFT, which detect mRNAs for CiFT1, CiFT2, and CiFT3 non-differentially (Nishikawa et al., 2007), and it is unclear which CiFT is expressed in bearing shoots; however, fruiting shoots are directly connected with fruits and CiFT expression might be enhanced by the same signal in fruits and fruit-bearing shoots.

**Flower number and CiFT expression**

Alternate bearing is a serious problem in citrus farming. To avoid alternate bearing, it is necessary to predict flower number as soon as possible and to adjust flowers to an appropriate number on the basis of the prospects. Because CiFT mRNA levels correlate with seasonal floral induction (Nishikawa et al., 2007), it was expected that CiFT mRNA levels also have a close correlation with the flower number the following spring. In the present study, we show that the CiFT expression in the stems from November to January closely correlated with flower number per sprouting node the following spring (Table 1). This result suggests that CiFT expression is one of the limiting factors of flower formation in annual flowering and that CiFT mRNA levels can be useful tools for predicting flower number. Changes in CiFT expression by the period of fruit bearing were similar to those of the flower number per node in each cultivar, but the correlation between the CiFT mRNA levels and the flower number seems different among cultivars (Fig. 2). To predict flower number by CiFT mRNA levels, clarification of the factors influencing this correlation are required.

Information about CiFT expression should be helpful for developing a technique to control flower number. In the present study, the correlation of fruit weight per leaf area was higher for CiFT expression (R² = 0.68 in the stem) than for flower number (R² = 0.39). This may be because dispersion is enhanced by factors other than the leaf-to-fruit ratio following CiFT expression, such as damage to trees from cold weather, defoliation, or the method of counting flower number. This correlation indicates that CiFT mRNA levels can correctly represent the effect of fruit bearing on flowering. Since flowering signals integrate FT in Arabidopsis (Komeda, 2004), CiFT expression may reflect the role of factors such as water stress and fertilization in addition to fruit bearing and temperature. By quantitatively analyzing CiFT expression rather than counting flower number, it will be possible to determine the effect of exogenous and endogenous factors on flowering more accurately at an earlier time point and to know the exact treatments necessary to adjust flower number.

Until now, alternate bearing has been evaluated by determining the annual changes of flower number or fruit production; however, this evaluation takes a long time and multiple factors affect the result. For these reasons, it is difficult to select cultivars that produce steady amounts of fruit every year. Since flowering suppression due to fruit bearing is one of the main reasons for alternate bearing, it is thought that the suppression of CiFT expression by fruits can be connected with alternate bearing. A further study about the relationship between fruit bearing and CiFT expression might indicate how non-alternate-bearing cultivars can be efficiently selected.

**Literature Cited**


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