RESEARCH PAPER

Increased CiFT abundance in the stem correlates with floral induction by low temperature in Satsuma mandarin (Citrus unshiu Marc.)

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Abstract

After several years in the juvenile phase, adult citrus trees show seasonal periodicity of flowering. A prolonged exposure to low temperature is one of the most important environmental cues for floral induction in citrus. In the present study, the expression of flowering-related genes during the annual cycle of flowering and inductive low-temperature treatment in Satsuma mandarin (Citrus unshiu Marc.) trees is investigated. Simultaneously, floral induction, which occurs before the period of morphological flower development, was estimated as the number of flowers after the forcing of sprouting by defoliation at 25°C. The expression of citrus FLOWERING LOCUS T homologues, CiFT, showed a seasonal increase during the floral induction period and was also induced by an artificial low-temperature treatment (15°C) at which floral induction occurred. By contrast, the mRNA level of CiFT did not show any distinct changes following a warm-temperature treatment (25°C) for 2.5 months, during which time floral induction was completely suppressed. Changes in the expression of the citrus homologues of TERMINAL FLOWER 1, LEAFY, and APETALA1 did not show any correlation with floral induction in the field or under artificial low-temperature conditions. In juvenile seedlings of Satsuma mandarin, which does not flower even under inductive low-temperature conditions, the mRNA levels of CiFT were not affected by the low-temperature treatment, unlike adult tissues. These results suggest that low temperature promotes floral induction via the activation of CiFT transcription in adult Satsuma mandarin trees and that, in the juvenile plant, CiFT transcription does not respond to low temperature.

Key words: Citrus, flowering, FT, low temperature, Satsuma mandarin.

Introduction

Flowering is one of the most crucial events in the plant life cycle. In many plant species, flowering is promoted by environmental conditions. One of the most important factors controlling flowering is the photoperiod, which is the duration of daily light. In Arabidopsis, genetic analyses showed that genes of CONSTANS (CO) and FLOWERING LOCUS T (FT) were involved in the photoperiodic induction of flowering (Koornneef et al., 1991). Another important factor controlling flowering is vernalization, which is the exposure to low temperature for several weeks. Without exposure to low temperature, the FLOWERING LOCUS C (FLC) represses flowering, and, at least in part, this occurs through repressing flowering activators, SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1) and FT (Michaels et al., 2005). By contrast, the expression of FLC is reduced by exposure to low temperature, and, subsequently, gene expression of the flowering activators can be induced. These results suggest that FLC plays an important role in the vernalization response of Arabidopsis (Henderson and Dean, 2004; Amasino, 2005). As with vernalization and photoperiod, ambient temperature ultimately affects flowering time and
expression of the floral pathway integrator FT in Arabidopsis (Blázquez et al., 2003; Samach and Wigge, 2005). The transition to flowering is delayed at a low ambient temperature (16 °C) and FVE and FCA, which are the genes in the autonomous pathway, have been proposed to communicate differences in ambient temperature. As a result, the expression of FT is down-regulated in Arabidopsis grown at 16 °C.

In woody perennials, the molecular mechanism for flowering has also been studied. Poplar, a species of deciduous tree, in the adult phase initiates seasonal floral induction during spring and summer, with an increase in the expression of its FT homologue (Bohlenius et al., 2006; Hsu et al., 2006). In these studies, it was reported that the expression of the FT homologue is regulated by the photoperiod (Hsu et al., 2006). On the other hand, in evergreen trees, the expression of FT has not been reported. Species belonging to the genus Citrus are evergreen trees and have interesting and distinct flowering behaviours. Most deciduous trees develop flower buds in the early summer and, subsequently, enter a prolonged winter rest before spring bloom. In contrast to deciduous trees, in evergreen citrus, low temperatures are one of the most important environmental cues to induce flowering (Davenport, 1990; Krajewski and Rabe, 1995). In subtropical climates, floral induction occurs from autumn to early winter. After the floral inductive period, citrus trees start the morphological development of floral buds in winter and then bloom in spring without a long rest period, which is typically observed in deciduous trees.

Inoue (1989, 1990) has reported that an ambient temperature of 25 °C is the threshold for floral induction in Satsuma mandarin (Citrus unshiu Marc.), a species of the genus Citrus. At temperatures above 25 °C, Satsuma mandarin trees continue vegetative growth without production of flower buds. By contrast, at temperatures below 25 °C, the trees produce flower buds. These results suggest that, in Satsuma mandarin, temperatures below 25 °C are effective for floral induction. These observations are valuable in the search for a molecular mechanism involved in the flowering of woody perennials because their flowering can be controlled by regulating the ambient temperature.

During floral induction in citrus, no morphological changes are observed in axillary buds that sprout floral or vegetative shoots in the subsequent spring (Krajewski and Rabe, 1995). In field conditions of Japan, vegetative and reproductive axillary buds of Satsuma mandarin are anatomically distinguishable after late December (Iwasaki, 1956). Inoue (1989, 1990) contrived a method to estimate the progress of floral induction before morphological flower development. In this method, potted trees are defoliated and transferred into a 25 °C room to force the buds to sprout. The number of flowers after the forcing of sprouting increased as the trees were exposed to low temperatures (15 °C) for longer periods. The number of flowers after the forcing of sprouting is assumed to show floral induction and degree of flowering at the time of defoliation and transfer to the 25 °C room. Therefore, this phenomenon is referred to as ‘flowering potential’ in this study.

The existence of juvenile phases in woody perennials has been a limiting factor for traditional breeding and genetic studies. Citrus has a long juvenile period, and trees do not flower until after 6–10 years. In sweet orange, Pillitteri et al. (2004a) reported that accumulation of transcripts of CstFL, a homologue of TERMINAL FLOWER1 (TFL1) in citrus, was positively correlated with juvenility and negatively correlated with expression of citrus LEAFY (LFY) and AP1 homologues. In citrus (hybrids between sweet orange and trifoliate orange), constitutive expression of LFY or AP1 genes derived from Arabidopsis dramatically reduced their generation time (Peña et al., 2001). Endo et al. (2005) previously generated transgenic trifoliate orange, in which an FT homologue, CiFT1, derived from Satsuma mandarin, was constitutively expressed. The 35S::CiFT1 trifoliate orange showed an extremely early flowering. These results suggest that FT, LFY, and AP1, which are key genes promoting flowering in Arabidopsis, could regulate the phase transition from the juvenile to the adult phases in citrus and its relatives. However, Endo et al. (2005) showed that effects on flowering were different among these key genes. Flowering of 35S::LFY and 35S::AP1 citrus was still under environmental control, showing seasonal periodicity regulated by factors other than expression of LFY and AP1. By contrast, the 35S::CiFT1 trifoliate orange developed flowers regardless of seasonal periodicity. Thus, it is considered that FT is more closely involved in seasonal periodicity than LFY and AP1.

However, the relationship between the seasonal periodicity of flowering and the expression of FT is not clear. To understand the molecular mechanism of the seasonal periodicity of flowering in citrus, it is important to investigate changes in endogenous mRNA abundance of key genes promoting flowering, such as FT, in relation to flowering potential.

In the present paper, the mRNA levels of flowering-related genes and flowering potential were monitored in adult Satsuma mandarin trees grown outdoors to investigate the molecular mechanisms underlying the seasonal periodicity of flowering. Moreover, to understand the effects of low temperature, the mRNA levels of flowering-related genes and flowering potential were investigated in trees grown at 15 °C and 25 °C. The results are the first of their kind, to our knowledge, to show the distinct relationships between flowering potential and mRNA levels of FT in evergreen woody perennials. This study is also the first, to our knowledge, to provide information on responsiveness to low
temperature in the expression of FT in woody perennials. On the basis of these results, the molecular mechanism underlying floral induction by low temperature in woody perennials is discussed.

Materials and methods

Plant materials

62-year-old ‘Miyagawa-wase’ Satsuma mandarin (Citrus unshiu Marc.) cultivated at the National institute of Fruit Tree Science (NIFTS), Okitsu (Shimizu-ku, Shizuoka, Japan) were used for gene isolation, Southern blotting, and analysis of gene expression during development. For the investigation of developmental changes in mRNA levels, leaves, stems, flowers at anthesis, whole fruits at 30 d after flowering (DAF), and peels and juice sacs at 60 DAF, 120 DAF, and 150 DAF were collected. In addition, seed and 4-month-old seedlings of ‘Aoshima’ Satsuma mandarin were also collected.

For the investigation of the seasonal changes in mRNA levels, 1-year-old potted ‘Aoshima’ Satsuma mandarin grafted on trifoliate orange cultivated at Okitsu were used. Spring shoots, which were sprouted and developed from a brunch of a tree in April, were harvested from June to next April. At each period, whole stems without leaves, which included internodes and nodes from the base to the apical part, and leaves without stems were collected from three individual plants for RNA extraction.

To investigate the effect of low temperature, 1-year-old potted ‘Okitsu-wase’ Satsuma mandarin trees grafted on trifoliate orange trees were purchased from a local market in early April. Flower induction by low-temperature treatment was performed according to Inoue (1990). The potted plants were grown outdoors until the beginning of the temperature treatment, during which spring flushes sprouted and completed hardening. The plants were transferred to rooms at 15 °C or 25 °C (low-temperature treatment or its control in adult plants, respectively) in early July and kept for 0, 0.5, 1, 1.5, 2, and 2.5 months, followed by complete defoliation; they were then placed at 25 °C for an additional 20 d to induce sprouting. As a control in juvenile plants, 4-month-old seedlings of ‘Aoshima’ Satsuma mandarin were kept at 15 °C for 2 months, defoliated, and transferred to a 25 °C room. The number and types of shoots formed, and days required for a first sign of flush visible after defoliation and transfer into a 25 °C room were counted. The trees were watered regularly, and the light conditions were the same as those outdoors. For RNA extraction, whole stems with leaves removed and leaves without stems were collected from three plants during the temperature treatment and after defoliation at 0.5-month and 0.1-month intervals, respectively. All samples for RNA extraction were frozen in liquid nitrogen immediately and stored at −80 °C until use.

DNA blot analysis

The genomic DNA of ‘Miyagawa-wase’ Satsuma mandarin was isolated from leaves by the method of Dellaporta et al. (1983). Ten μg of total DNA of Satsuma mandarin was completely digested with HindIII, EcoRI, Dral, or BamHI. A 0.65 kb fragment of the CiFT1, which covers from the 5′ end to the EcoRV site in the 3′ untranslated region, was used as a probe. Digested DNA was electrophoresed on a 1.0% agarose gel and blotted onto a nylon membrane (Hybond-NX, Amersham Bioscience, Little Chalfont, UK). Probe labelling by DIG, hybridization, and detection were conducted according to the manufacturer’s instructions (Roche Diagnostics GmbH Mannheim, Germany).

Isolation and sequence analysis of CiFT2 and CiFT3

The first-strand cDNA was synthesized from 1 μg of the total RNA isolated from leaf, flower, fruit, and low-temperature-treated stem of ‘Miyagawa-wase’ Satsuma mandarin with the Ready-To-Go You-Primed First-Strand Kit (Amersham Bioscience). The RT-PCR reaction was performed with the primers designed on the basis of the CiFT1 sequence (Table 1). The amplified cDNAs were cloned into a pCR-TOPO vector with a TOPO TA Cloning Kit (Invitrogen, San Diego, CA). Sequences were determined with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using the ABI PRISIL 3100 Genetic Analyser (Applied Biosystems). After sequence analysis, RACE-PCRs for the new CiFT clones, CiFT2 and CiFT3, were performed with the cDNA template created from fruits and chilling-treated stems of Satsuma mandarin, respectively, using the SMART RACE cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA). The primer sets used for RACE-PCR are shown in Table 1. The amplified cDNAs of the 5′ and 3′ ends for CiFT2 and CiFT3 were cloned and sequenced. A phylogenetic tree was constructed by Genetyx-Win v.4.0.1 (Software Development Co., Tokyo, Japan) by the UPGMA method. Sequence data from this article have been deposited with the GenBank data libraries under accession numbers AB301934 and AB301935.

Total RNA extraction and real-time quantitative RT-PCR

Total RNA was extracted according to the method described by Ikoma et al. (1996). For real-time RT-PCR analysis, the total RNA was cleaned up with the RNeasy Mini Kit (Qagen, Hilden, Germany) with on-column DNase digestion. The reactions of reverse transcription (RT) were performed with 0.2 μg of purified total RNA and a random hexamer at 37 °C for 60 min using TaqMan Reverse Transcription Reagents (Applied Biosystems).

TaqMan MGB probes and sets of primers for CiFT1, CiFT2, CiFT3, total CiFT, CiSTF1, CiSFTL, CiLFY, and CiSAP1 were designed with the Primer Express software (Applied Biosystems; Table 1). 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 ABI PRISIL 7000 (Applied Biosystems) according to the manufacturer’s instructions. Each reaction contained 900 nM primers, a 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 analysed with ABI PRISIL 7000 Sequence Detection System Software (Applied Biosystems) and normalized with the results of 18S ribosomal RNA. Real-time quantitative RT-PCR was performed in three replicates for each sample and data were indicated as means ± SD (n=3).

Results

Isolation and characterization of novel CiFT homologues

To estimate the number of CiFT copies in the genome, genomic DNA hybridization was performed. Digestion by four restriction enzymes indicated that there are several copies of the FT homologue in Satsuma mandarin (data not shown). The primers were designed on the basis of the sequence of CiFT cDNA (accession No. AB027456) to amplify other CiFT homologues (Table 1). Fifty-two
clones encoding CiFT were obtained for cDNAs synthesized from leaf, flower, fruit, or low temperature-treated stem. From these clones, two novel homologues were identified (Fig. 1). In this study, the original CiFT, which was found in an EST catalogue of mature citrus fruit, was also isolated from fruit cDNA and renamed CiFT1. Two novel homologues were designated as CiFT2 and CiFT3 (Fig. 1). CiFT2 and CiFT3 were isolated from the cDNAs of fruit and the low temperature-treated stem, respectively. CiFT1 and CiFT2 were also cloned from flower cDNA. From leaves, all three CiFTs were cloned. These three CiFTs showed high identities (>95% at the amino acid sequence level) with each other (Fig. 1A). The single amino acid residue, Tyr-85 in Arabidopsis FT, which has been reported as a key amino acid residue to promote flowering (Hanzawa et al., 2005), was conserved among CiFTs and primers can detect mRNA for CiFT in their translated region (Table 1). Since this set of probe and primer sets were designed on the basis of the differences in the nucleotide sequence at 5’ untranslated regions and primer sets were designed on the basis of the differences in the nucleotide sequence at 5’ untranslated regions. To detect the mRNA for the three homologues differentially, TaqMan MGB probes and primers were designed on the basis of the differences in the nucleotide sequence at 5’ untranslated regions (Table 1). In addition, a set of TaqMan MGB probe and primer sets were designed on the basis of the conserved nucleotide sequence among three homologues in their translated region (Table 1). Since this set of probe and primers can detect mRNA for CiFT1, CiFT2, and CiFT3 undifferentially, the target for detection in this case was designated as total CiFT. For the detection of CsTFL, CsLFY, or CsAP1 (citrus homologue of TFL1, LFY, or AP1, respectively), the TaqMan probe and primer sets were synthesized on the basis of the conserved sequence between each homologue of Satsuma mandarin and trifoliate orange (Table 1).

The mRNA for CiFT1 and CiFT2 were detected strongly in the later stages of fruit development; on the other hand, they were detected slightly in seed and flower and scarcely in stem, leaf, and immature fruit (Fig. 2). The CiFT3 gene was expressed only in the stem and leaf tissues. No mRNA accumulation for CiFT3 was detected in any other tissues. Changes in the mRNA accumulation for total CiFT showed similar patterns to those of CiFT1.

### Table 1. Primers sequences used for the PCRs and TaqMan MGB probes and primer sequences used for the real-time quantitative RT-PCRs of flowering-related genes

<table>
<thead>
<tr>
<th>Target</th>
<th>Sequences (5’ to 3’)</th>
<th>Primer or probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CiFT2 and CiFT3</td>
<td>TTTGTTGTTGATGTTCTTGAC</td>
<td>Sense primer</td>
</tr>
<tr>
<td>CiFT1</td>
<td>CATCGCTCTCCTGAGTGCCG</td>
<td>Sense primer</td>
</tr>
<tr>
<td>CiFT2 and CiFT3</td>
<td>TCTGACTCTGTGACACACCTAG</td>
<td>Sense primer</td>
</tr>
<tr>
<td>CiFT3</td>
<td>CATCCTCTCCCTGGCATGATCATA</td>
<td></td>
</tr>
<tr>
<td>Total CiFT</td>
<td>GGCCTTAGGGAGATTTGCCAGT</td>
<td></td>
</tr>
<tr>
<td>CsTFL1</td>
<td>AAGGGGACATCTCC</td>
<td></td>
</tr>
<tr>
<td>CsLFY</td>
<td>CCAAGGAGCGCGC</td>
<td></td>
</tr>
<tr>
<td>CsAP1</td>
<td>ATAGAGGAGCAAACA</td>
<td></td>
</tr>
</tbody>
</table>

* Target for amplification or detection.
The mRNA for CsTFL accumulated abundantly in the aerial parts of the seedlings and flowers. However, low amounts of the mRNA were observed in other tissues. The mRNA abundance of CsLFY showed high levels in the juice sacs of fruit through all developmental stages investigated. In the aerial parts of the seedlings, adult stems and flowers, the transcripts of CsLFY accumulated distinctly, although the levels were less than those in juice sacs. The mRNA levels of CsAP1 were much higher in the stem and peel of mature fruit than they were in other tissues.

To understand the molecular mechanism of seasonal periodicity of flowering, seasonal changes in the flowering potential and the mRNA levels of flowering-related genes were investigated with 1-year-old adult Satsuma mandarin trees, which had been prepared by picking a shoot from the adult Satsuma mandarin tree, grafting it onto a trifoliate orange root stock, and growing it for a year in the field. Throughout an experimental period, the adult 1-year-old
Satsuma mandarin trees were potted and grown in the field. In April, spring shoots sprouted on the 1-year-old Satsuma mandarin trees, and their growth ceased in June. Sampling of the spring shoots and leaves was started just before their growth cessation in June for mRNA analysis. The estimation of the flowering potential was started after September by the defoliation method (Inoue, 1989, 1990, see Materials and methods).

When trees were defoliated and forced in September, no flower sprouting was observed (Fig. 3). After October, flowers could be observed following defoliation of the trees and forcing. In November, the number of flowers increased, with large variation between individuals. At that time, floral induction was in its early stages. It may be that the nutritional conditions affect the beginning of floral induction in each tree and cause large differences in the number of flowers among the replicate plants. Since the number of flowers increased significantly from 15 October

![Fig. 2. Expression of CiFT1, CiFT2, CiFT3, total CiFT, CsTFL, CsLFY, and CsAP1 in various tissues of Satsuma mandarin: seed, aerial part of seedling (a), adult stem (s), and adult leaves (l) collected in June, flower at anthesis (f), fruit at 30 d after flowering (DAF), juice sacs at 60, 120, and 150 DAF, and peel at 60, 120, and 150 DAF. The levels of gene expression were analysed by TaqMan real-time quantitative RT-PCR. The TaqMan MGB probes and sets of primers used for the analyses are shown in Table 1. Data are means ± SD (n=3).](https://academic.oup.com/jxb/article-abstract/58/14/3915/460726)

![Fig. 3. Changes in flowering potential of the plants grown in the field. At the date indicated, three potted trees were defoliated and transferred to a 25 °C room. This treatment was employed to force the buds to sprout. The number of flowers was counted for 20 d after the forcing of sprouting. Data are means ± SD (n=3).](https://academic.oup.com/jxb/article-abstract/58/14/3915/460726)
to 15 November, it was deduced that floral induction occurred, and after that, the flowering potential increased.

In stem tissues, no changes were observed in the mRNA level of \textit{CiFT1} throughout the experimental period (Fig. 4A). On the other hand, that for \textit{CiFT2} increased just before and during flower sprouting (March and April). The transcripts of \textit{CiFT3} were at very low levels during summer, but began to increase after September with the decrease in the ambient temperature in the autumn. The mRNA levels of \textit{CiFT3} peaked in January, after which the mRNA levels decreased rapidly towards spring. The expression pattern for \textit{total CiFT} was very similar to that for \textit{CiFT3}. These results indicate that the increases in \textit{CiFT3} and \textit{total CiFT} mRNA in stems occur during floral induction. The mRNA abundance of \textit{CsLFY} decreased towards winter and increased just before and during flower sprouting (March and April) with the increase in the ambient temperature. The transcript levels of both \textit{CsTFL} and \textit{CsAP1} were highest in June, but they were extremely reduced in July and maintained at low levels until April.

The gene expression in leaves is shown in Fig. 4B. The mRNA levels of \textit{CiFT1}, \textit{CiFT3}, and \textit{total CiFT} began to increase after November, peaked in January, and then decreased towards spring. \textit{CiFT2} was expressed at high levels in the summer and then decreased towards winter. It increased slightly in March. No transcript accumulation of \textit{CsTFL} was detected. That for \textit{CsLFY} maintained low levels during summer and then increased in March. For \textit{CsAP1}, the mRNA level was high in June and April but low in January.

**Effect of low-temperature treatment**

One-year-old adult Satsuma mandarin trees, which had been prepared as described above, were used to clarify the

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**Fig. 4.** Expression of \textit{CiFT1}, \textit{CiFT2}, \textit{CiFT3}, \textit{total CiFT}, \textit{CsTFL}, \textit{CsLFY}, and \textit{CsAP1} in stems (A) and leaves (B) of Satsuma mandarin harvested throughout a year. The levels of gene expression were analysed by TaqMan real-time quantitative RT-PCR. The TaqMan MGB probes and sets of primers used for the analyses are shown in Table 1. Data are means ±SD (n=3). Changes in average air temperature under field conditions during the experimental periods in 2005 to 2006 are shown in the lower-left graph.
effects of low temperature on the floral induction and expression of flowering-related genes. As described above, the 1-year-old adult Satsuma mandarin trees were grown in the field until late June, when the growth of spring shoots ceased. Subsequently, the trees were grown in rooms at 15°C (for the low-temperature treatment) or 25°C (for the high-temperature treatment) from late June. The trees grown at 15°C were defoliated and forced at 25°C to estimate the flowering potential at 0, 0.5, 1, 1.5, 2, and 2.5 months after the beginning of the low-temperature treatment. The trees grown at 25°C were defoliated and forced at 25°C as a control. Samples (stems and leaves) for mRNA analysis were collected at the time of defoliation. Moreover, from the trees treated with low or high temperature for 2.5 months, the stems were sampled at 3 d and 6 d after the beginning of the forcing treatment.

Vegetative shoots sprouted until August on the trees grown at 25°C, but not on the trees grown at 15°C. The results for the numbers and types of shoots produced after forcing are summarized in Table 2. The number of vegetative shoots was similar for the 15°C and 25°C treatments. Flower sprouting after forcing was observed on trees which had grown at 15°C for more than 1.5 months. This result confirms that the floral induction of Satsuma mandarin occurred as a result of the low-temperature treatment at 15°C for 1.5 months (Inoue, 1990). As the length of the low-temperature treatment increased, the number of flowers sprouting after forcing increased. On the other hand, flower sprouting after forcing was not observed on trees grown at 25°C. This result confirmed that floral induction of Satsuma mandarin did not occur at high temperature (25°C) throughout the present experimental period (Inoue, 1990).
The expression of CiFT1, CiFT2, CiFT3, and total CiFT was greatly induced in the stem by prolonged exposure to low temperature (Fig. 5A). The mRNA levels of CiFT1, CiFT3, and total CiFT in low temperature-treated trees increased greatly after 1.5 months, when floral induction occurred, after which levels remained high under low temperature. However, those levels declined strikingly after the forcing treatment. CiFT3 and total

Table 2. Flowering potential of the plants after the temperature treatments at 15 °C and 25 °C from late June

<table>
<thead>
<tr>
<th>Periods kept in 15 °C or 25 °C rooms (months)</th>
<th>15 °C</th>
<th></th>
<th>25 °C</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leafless flower</td>
<td>Leafy flower</td>
<td>Total</td>
<td>No. of vegetative shoots</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>20.0 (100)</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>11.3 (100)</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>19.3 (100)</td>
</tr>
<tr>
<td>1.5</td>
<td>3.7</td>
<td>13.0</td>
<td>16.7 (38.2)</td>
<td>12.7 (61.8)</td>
</tr>
<tr>
<td>2</td>
<td>8.7</td>
<td>27.0</td>
<td>35.7 (71.5)</td>
<td>12.7 (28.5)</td>
</tr>
<tr>
<td>2.5</td>
<td>21.3</td>
<td>27.7</td>
<td>49.3 (69.4)</td>
<td>18.7 (31.6)</td>
</tr>
</tbody>
</table>

*Figures in parentheses represent percentages of flowers or vegetative tissues relative to total number of sprouting buds.

Fig. 5. Expression of CiFT1, CiFT2, CiFT3, total CiFT, CsTFL, CsLFY, and CsAP1 in stems (A) and leaves (B) subjected to temperature treatments of 15 °C (closed diamonds: adult tissues; open circles: juvenile plant tissues) or 25 °C (closed squares: adult tissues). The levels of gene expression were analysed by TaqMan real-time quantitative RT-PCR. The TaqMan MGB probes and sets of primers used for the analyses are shown in Table 1. Data are means ± SD (n=3). The arrows indicate the time points at which plants began to form flower buds after defoliation and transfer from 15 °C into 25 °C.
CiFT showed similar changes in the mRNA expression in this experiment as well. The mRNA levels of CiFT2 increased within 1 month after transfer to 15 °C and generally remained high. In the high-temperature (25 °C) treatment, continuous changes were not distinctly observed in the mRNA levels of any CiFTs in the stems. The transcripts of CsLFY in stem tissue were up-regulated by exposure to low temperature for 2.5 months and remained at high levels even after the forcing treatment. No distinct changes caused by the low-temperature treatment were observed in the mRNA levels of CsTFL and CsAP1.

In leaves exposed to low temperature, the mRNA levels of CiFT3 and total CiFT increased gradually during the experimental period, while those in leaves exposed to 25 °C remained low (Fig. 5B). No distinct changes were observed in the expression of CiFT1 and CiFT2. The mRNA level of CsTFL was undetectable in both temperature treatments throughout the present experimental period. The transcript levels of CsLFY in low-temperature-treated leaves maintained similar levels to those in leaves treated at 25 °C. The mRNA level of CsAP1 was not affected by any temperature treatment in leaves.

In addition, 4-month-old seedlings of Satsuma mandarin were subjected to the low-temperature treatment. In seedlings, flowering did not occur because of their juvenility. The changes in the gene expression were investigated with stems or leaves at 0, 0.5, 1, and 2 months after transfer to 15 °C (Fig. 5). At 0 months, the level of mRNA for CsTFL was much higher in the stem of seedlings than in those of adult trees, although the mRNA level of the seedlings decreased just after transfer to 15 °C. The level of CsTFL mRNA in the leaves of seedlings was low during the exposure to 15 °C. The transcripts of CiFTs, CsLFY, and CsAP1 remained at low levels during the experimental periods for both stems and leaves of seedlings.

### Discussion

**Relationship between seasonal floral induction and CiFT expression in the adult phase**

The role of temperature on periodic floral induction in adult citrus trees has been well researched (Davenport, 1990; Krajewski and Rabe, 1995). Temperature below 25 °C favours floral induction in Satsuma mandarin (Inoue, 1990). In the present study, floral induction was observed in the low-temperature (15 °C) treatment, but not in the high-temperature (25 °C) treatment (Table 2). Thus, controlling flowering by means of a temperature treatment had succeeded. In trees in which the flowering was artificially controlled, the relationship between periodic floral induction and expression of flowering-related genes could be investigated.

In adult trees, the expression of total CiFT in stem and leaf tissues was enhanced by the low-temperature treatment, and accompanied an increase in the flowering
potential (Fig. 5; Table 2). The changes in the expression of other flowering-related genes, CsTFL, CsLFY, and CsAP1, were not consistent with that of flowering potential. Under field conditions, it was observed that the flowering potential started to increase from mid-October (Fig. 3), accompanying the increases in the mRNA level of total CiFT from September to January in stem tissue (Fig. 4). Thus, these results indicate an association between the increase in the expression of CiFTs and floral induction by low temperature. Recently, it has been shown that FT homologues have broader roles in plant development, not only in flowering induction but also in growth attenuation. In conifers, the expression of an FT homologue is correlated with seasonal cycles of growth and no growth (bud set and bud outgrowth) in juvenile plants (Gyllenstrand et al., 2007). Lifschitz and Eshed (2006) also proposed that the primary targets for both FT- and TFL1-like genes in angiosperms may actually be the induction and termination of growth. Therefore, CiFTs expression in the stem might be associated with growth rhythms.

In the present study, three homologues of CiFT were investigated in Satsuma mandarin. Endo et al. (2005) previously reported that CiFT1 is capable of inducing extremely early flowering and fruiting in trifoliate orange by ectopic expression. The sequences of CiFT2 and CiFT3 showed high identity with that of CiFT1 and the key amino acid residue, which determines the biochemical function for flowering (Hanzawa et al., 2005), was conserved in three CiFTs and Arabidopsis FT, suggesting that CiFT2 and CiFT3 could be activators of flowering, as is CiFT1.

CiFT1 and CiFT2 were expressed strongly in fruit tissues but scarcely in stem and leaf tissues (Fig. 2). On the other hand, CiFT3 was expressed relatively highly in stem and leaf tissues but scarcely in fruit tissues. In stem and leaf tissues, the pattern of expression of total CiFT is more similar to that for CiFT3 than to those for CiFT1 and 2 (Figs 4, 5). These results suggest that, in stem and leaf tissues, mRNA abundance of CiFT3 is much higher than that of CiFT1 and 2. There are several reports of the number of FT-like genes identified in one species. For example, eight FT-like genes were expressed in leaves of rice (Izawa et al., 2002), and in conifers at least two FT homologues were expressed (Gyllenstrand et al., 2007). In citrus, numerous FT-like genes were estimated by Southern blot analysis (data not shown). The present study identified three transcribed FT-like genes, which might not be all of the FT homologues expressed in citrus. However, CiFT3 is considered to play a principal role in floral induction in citrus.

In Arabidopsis and rice, protein of FT and its orthologue in rice, Hd3a, was shown to be produced in the vascular tissues of leaves and to move from the leaves to the shoot apices as a mobile flowering signal (Takada and Goto, 2003; Corbesier et al., 2007; Tamaki et al., 2007). In tomato, it was demonstrated that SFT, the FT homologue of tomato, was expressed in leaves and that graft-transmissible SFT signals complemented all aspects of the sft phenotype (Lifschitz et al., 2006). The expression of total CiFT was also induced in citrus leaves during winter under field conditions or by the low-temperature treatment. However, changes in the flowering potential both under field and low-temperature conditions exhibited a closer relationship with the expression of CiFT in stems than with that in leaves. In citrus, the role of expression of CiFT in leaves is less clear. It is also unclear whether CiFT expressed in leaves moves to axillary buds, where floral induction occurs. Previously, a specific effect of low temperature on floral induction was observed in citrus cultured buds (García-Luis et al., 1992). The results suggest that buds could sense the low temperature by themselves without leaves. Further research will be needed to understand whether or how the CiFT expression in leaves affects floral induction in citrus.

In Arabidopsis, flowering time is delayed at low ambient temperatures (Blázquez et al., 2003; Samach and Wigge, 2005). Recently, Arabidopsis SHORT VEGETATIVE PHASE (SVP) was demonstrated to mediate the temperature-dependent functions of FCA and FVE within the thermosensory pathway (Lee et al., 2007). SVP controls flowering time by negatively regulating the expression of FT via direct binding to the CArG motifs in the FT sequence. Thus, CiFTs in citrus might be regulated by homologues of such genes, whereas citrus flowering is induced by low temperature.

CiFT expression is correlated with juvenility in citrus

In the present paper, it has been demonstrated that the mRNA levels of CiFT were low and not affected by the low-temperature treatment in juvenile tissues, in contrast to the response of adult tissues (Fig. 5). These results indicate that juvenile trees are not responsive to low temperature, in terms of CiFT transcription. The transcripts of CsTFL accumulated at a higher level in juvenile plants than in adult ones, which is consistent with the results obtained by Pillitteri et al. (2004a). However, the level decreased within 0.5 months after transfer to low temperature (Fig. 5). The reduced levels were as low as those observed in the adult tissues. These results suggest that, even during the juvenile period, the mRNA level of CsTFL can decrease to the same level as that during the adult period and that the decreased expression level for CsTFL by itself may not be the main cause in juvenility. The repression of floral induction in juvenile plants might occur as a result of the lack of response of CiFT transcription to low temperature although monitoring of seasonal expression patterns of the CiFT gene as well as
other flowering genes is required to clarify why juvenile plants are not able to flower.

**Axillary bud development**

The expression of CsTFL, CsLFY, and CsAPI did not seem to be correlated the floral induction in response to low temperature in citrus. TFL1 is a promoter of vegetative identity in the shoot meristems of Arabidopsis. Under field conditions, the transcripts of CsTFL were detected only in developing vegetative shoots in June (Fig. 4). This result suggests that CsTFL might affect the vegetative growth in an adult citrus tree.

The mRNA level of CsLFY in the stem tissue increased gradually during spring (from January to April) under field conditions (Fig. 4) and rapidly following exposure to low temperature for 2.5 months (Fig. 5). In the spring, flower buds develop morphologically in Satsuma mandarin grown outdoors. At 2.5 months of artificial low-temperature treatment, a few flower buds were visible. Thus, the increase in CsLFY transcripts coincided with floral-bud development both under field and low-temperature conditions. This suggests that CsLFY may play a role in flower-bud sprouting and/or flower-bud development after floral induction by CiFT transcription. The result agrees with previous works, in which expression of CsLFY in sweet orange was induced by low-temperature (15°C) treatment for 2 months (Pillitteri et al., 2004a).

In Arabidopsis, AP1 is regulated by FT and LFY (Ruiz-Garcia et al., 1997). Constitutive expression of AP1 in transgenic Arabidopsis revealed that AP1 plays a role in floral induction (Mandel and Yanofsky, 1995). In sweet orange, Pillitteri et al. (2004a, b) reported an increase in the CsAP1 transcript at the end of the floral induction period and suggested the involvement of CsAP1 in floral organ development. The present work showed no distinct change in CsAP1, a discrepancy which may result from genotypic differences between sweet orange and Satsuma mandarin.

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