Expression of Flower Promoting Genes in Sweet Orange during Floral Inductive Water Deficits

Eduardo J. Chica¹ and L. Gene Albrigo²

Citrus Research and Education Center, University of Florida, IFAS, 700 Experiment Station Road, Lake Alfred, FL 33850

ADDITIONAL INDEX WORDS. floral induction, transcript accumulation, *CsFT*, *CsSLI*, *CsAPI*, *CsLFY*, subtropical

ABSTRACT. Cool ambient temperatures (5 to 20 °C) and water deficit are the only factors known to induce flowering in sweet orange (*Citrus sinensis*). Whereas the effects of cool ambient temperatures on flowering have been described extensively, reports on the mechanisms underlying floral induction by water deficit in sweet orange (and other tropical and subtropical species) are scarce. We report changes in the accumulation of transcripts of four flower-promoting genes, *CsFT*, *CsSLI*, *CsAPI*, and *CsLFY*, in sweet orange trees in response to water deficit or a combination of water deficit and cool temperatures under controlled conditions. Exposure to water deficit increased the accumulation of *CsFT* transcripts, whereas transcripts of *CsSLI*, *CsAPI*, and *CsLFY* were reduced. However, when water deficit was interrupted by irrigation, accumulation of *CsFT* transcripts returned rapidly to pre-treatment levels and accumulation of *CsSLI*, *CsAPI*, and *CsLFY* increased. The accumulation of *CsFT* transcripts in trees during the combined water deficit and cool temperatures treatment was higher than in trees exposed to either factor separately, and accumulation of *CsAPI* and *CsLFY* transcripts after the combined treatment was also higher. These results suggest that water deficit induces flowering through the upregulation of *CsFT* and that *CsFT* is the leaf integrator of flower-inducing signals generated by the exposure to water deficit and cool temperatures in sweet orange.

Under natural conditions, citrus (*Citrus* sp.) trees are induced to flower by seasonal exposure to either cool ambient temperatures (5 to 20 °C) or water deficit (Cassin et al., 1969; Moss, 1969). In subtropical regions where citrus are grown, cool temperatures during the winter induce flowering the next spring, whereas in tropical regions, flowering is induced during the dry season with flowering after the first effective rains of the rainy season (Cassin et al., 1969). For both stimuli (cool temperatures and water deficit), the intensity of the induced flowering depends on both the time of exposure to the inductive stimuli and the intensity at which the stimuli was present (Cassin et al., 1969; Southwick and Davenport, 1986). For cool temperatures, the maximum flowering intensity in sweet orange has been reported to be obtained after 8 weeks of exposure to temperatures between 10 and 15 °C (Moss, 1969). For water deficit, no comparable studies have been reported in sweet orange, but exposure to moderate or severe water deficit for 5 weeks in ‘Tahiti’ lime (*Citrus latifolia*) produced maximum flowering intensity compared with shorter exposures and milder deficit (Southwick and Davenport, 1986). Under field conditions in a humid subtropical climate, exposure to 60 to 75 d of drought has been related to optimum flowering (Albrigo et al., 2006).

In arabidopsis (*Arabidopsis thaliana*), several mechanisms for the regulation of seasonal flowering have been identified during the last 15 years (see Amasino, 2010, for a review). In these proposed mechanisms, the genes *Flowering locus T* (*FT*), *Apetalal* (*API*), *Leafy* (*LFY*), and *Suppressor of overexpression of Constans 1* (*SOC1*) play a pivotal role either integrating signals from flowering-promoting or flowering-inhibiting stimuli [*FT* or *SOC1* (Abe et al., 2005; Corbesier et al., 2007; Lee

and Lee, 2010; Samach et al., 2000)] or initiating the morphogenesis of floral organs in the shoot meristem [*API* and *LFY* (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995)]. In citrus, putative orthologs of these genes have been isolated and characterized (Nishikawa et al., 2007; Pillitteri et al., 2004b; Tan and Swain, 2007). Exposure to cool floral-inductive temperatures increased the expression (transcript accumulation) of *CsFT* (Nishikawa et al., 2007), which is consistent with the function of *FT* in arabidopsis as the ultimate flowering-promoting signal (Abe et al., 2005). In contrast, expression of *CsAPI* and *CsLFY* increased only toward the end of a floral-inductive temperature treatment (Pillitteri et al., 2004a), which is consistent with their function in arabidopsis as floral meristem identity genes (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995) and with earlier reports of the microscopical differentiation of floral meristems in citrus only after floral induction is over (Abbot, 1935). Although the patterns of expression of *CsSLI* (the ortholog of arabidopsis *SOC1*) have not been reported in response to floral inductive stimuli, the conservation of its function in citrus as a floral promoter is supported by the early flowering phenotype of arabidopsis *SOC1* mutants constitutively expressing *CsSLI* (Tan and Swain, 2007). In arabidopsis, *SOC1* integrates signals from the photoperiod, vernalization, gibberellin, and autonomous floral regulatory pathways (Lee and Lee, 2010).

Although *CsFT*, *CsAPI*, *CsLFY*, and *CsSLI* are involved in the regulation of flowering in citrus, the specific mechanisms regulating the expression of these genes must have evolved in citrus to respond to stimuli different to those inducing flowering in arabidopsis based on differences in their flowering response. In arabidopsis, plants are induced to flower by the transition from short to long photoperiods and by treatment with gibberellins under non-inductive short days (Blázquez et al., 1998); citrus flowering, however, can be considered mostly photoperiod insensitive (Moss, 1969), and gibberellins inhibit rather than promote flowering (Monselise et al., 1964). Furthermore,

Received for publication 1 Oct. 2012. Accepted for publication 10 Jan. 2013.

¹Current address: Carrera de Ingeniería Agrícola y Biológica, Escuela Superior Politécnica del Litoral, Km. 30.5 vía perimetral, Guayaquil, Ecuador.

²Corresponding author. E-mail: albrigo@ufl.edu.

whereas in citrus, temperatures between 5 and 20 °C induce flowering (Moss, 1969), in arabidopsis, flowering could be induced by higher (greater than 23 °C) ambient temperatures (Balasubramanian et al., 2006; Blázquez et al., 2003) or enabled by low vernalizing (less than 5 °C) temperatures (see Kim et al., 2009, for a review). In addition, no effects of water deficit as a floral-inductive factor have been reported for arabidopsis, whereas in citrus (and other subtropical and tropical species), water deficit is a key floral inductive factor (Albrigo and Galen-Saúco, 2004).

The objective of this work was to determine the expression patterns of *CsFT*, *CsAPI*, *CsLFY*, and *CsSLI* under floral-inductive water deficit and a combination of cool floral-inductive temperatures and water deficit in sweet orange trees; these two conditions are typical of the floral-inductive periods in humid subtropical and tropical climates where citrus is grown.

Materials and Methods

PLANT MATERIAL AND EXPERIMENTAL CONDITIONS. The experiments were conducted using two- to three-year-old potted (12-L pots) ‘Washington Navel’ sweet orange rooted cuttings (≈ 1 m tall) with fully matured foliage (no tender shoots). Before the start of the experiments, trees were kept in a greenhouse with a minimum temperature of 23 °C (maximum temperature was 31 °C) and watered every 2 d to saturation. While in this greenhouse, trees were exposed to natural variations in photoperiod (12.5/11.5 to 11/13 h, day/night) and photosynthetic photon flux (*PPF*) between 1210 and 1340 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Tree water status was monitored before and throughout the experiments by measuring the midday stem water potential (ψ_s) using the pressure chamber method (Scholander et al., 1965) on leaves covered in an aluminized bag for at least 1 h before measurement (McCutchan and Shackel, 1992). The midday stem water potential of the trees before the beginning of the experiment was -1.1 ± 0.10 MPa.

Two experiments were conducted at separate times: the first experiment tested the effect of floral-inductive water deficit only on the expression of *CsFT*, *CsAPI*, *CsLFY*, and *CsSLI*; whereas the second experiment tested the effect of floral-inductive water deficit and floral-inductive cool temperatures on the expression of the same genes. Both experiments were conducted in temperature-controlled growth rooms illuminated with fluorescent lights (800 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ *PPF* at the canopy level) with a 11/13-h (day/night) photoperiod. In the first experiment, trees were exposed to floral-inductive water deficit at a constant non-floral-inductive temperature (23 ± 1 °C) for 60 d, whereas in the second experiment, trees were exposed to floral-inductive water deficit at a constant floral-inductive temperature (12 ± 1 °C) for 40 d. After each treatment was over, trees were transferred back to the greenhouse and irrigation was restored to promote bud sprouting.

Water deficit was imposed by withholding irrigation until ψ_s reached -2.0 ± 0.12 MPa (moderate water deficit) and then kept at this level by irrigating the trees with a volume of water that matched the daily weight loss of the trees. This level of water deficit (≈ -2 MPa) was reached between Days 15 and 20 after the beginning of the experiment. The cool temperature treatment was applied by setting the temperature of the growth room at 12 °C at the beginning of the experiment.

SAMPLE COLLECTION, CONSERVATION, AND RNA EXTRACTION. Samples for gene expression analysis were collected at 10-d

intervals starting on the day when the treatments were applied. *CsFT* expression was measured in leaves, whereas *CsAPI*, *CsLFY*, and *CsSLI* were measured in axillary buds; this choice of tissues was based on the most likely site of functional expression of these genes inferred from works in arabidopsis (Corbesier et al., 2007; Wigge et al., 2005) and citrus (Furr et al., 1947; García-Luís and Kandušer, 1995; García-Luís et al., 1989). Leaf and bud samples were randomly collected from the three most apical nodes in mature six- to seven-node-long shoots because flowering is most likely to occur at these positions (Sauer, 1954; Valiente and Albrigo, 2004). At each time, six shoots were sampled on each tree; thus, leaf and bud samples consisted of a pool of six leaves or buds per tree. All samples were collected between 1445 and 1515 HR local standard time (Lake Alfred, FL), immediately frozen in liquid nitrogen, and stored at -80 °C until used.

Total RNA was extracted from the collected samples using a phenol–chloroform precipitation method (Chomczynski and Sacchi, 1987) and purified using silica membranes (Epoch Biolabs, Sugar Land, TX) with on-column DNase digestion (Qiagen, Germantown, MD). RNA quality was evaluated by spectrophotometry and denaturing gel electrophoresis and quantified by spectrophotometry (Sambrook et al., 1989).

QUANTITATIVE REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION. Five hundred nanograms of total RNA were used for cDNA synthesis in a 20- μL reaction with oligo dT primers (SuperScriptIII[®]; Invitrogen, Carlsbad, CA). One microliter of the synthesized cDNA was used for two-step (95 °C denaturation and 60 °C for 1 min annealing and extension) quantitative polymerase chain reaction (qPCR) in a 20- μL reaction using SYBR green detection (SYBR[®] Premix ExTaq[™]II; Takara, Mountain View, CA) on an Applied Biosystems 7500 FAST real-time PCR system (Life Technologies, Carlsbad, CA). Primers for qPCR were: 5'-CGGCGGAAGGACTATGAC-3' and 5'-TGTGAGAAAGCCAGAGAGGAA-3' (*CsFT*), 5'-CAGCCAGAGAATCTAACAACG-3' and 5'-TCAGTTTTGTGGTGGTATTGCC-3' (*CsSLI*), 5'-CCCTGGAGTGCAAC AACCT-3' and 5'-CTGATGTGTTTGAGAGCGGT-3' (*CsAPI*), and 5'-TCTTGATCCAGGTCCAGAACATC-3' and 5'-TAGTCACCTTGGTTGGGCATT-3' (*CsLFY*). *CsGAPDH* was used as reference gene (5'-GGAAGGTCAAGATCGCA ATCAA-3' and 5'-CGTCCCTCTGCAAGATGACTCT-3'). All qPCR assays were validated for specific amplification and optimized for amplification efficiencies between 1.88 and 2.05 with a linear dynamic range of six log10 cycles before the experiments. The sequence of the primers to amplify *CsLFY* was obtained from Nishikawa et al. (2009), whereas all other primer sequences were designed in-house. *CsFT* primers on this study were designed based on the sequence of *CiFT3* reported by Nishikawa et al. (2009). The *CiFT3* sequence was selected over *CiFT2* or *CiFT* because, reportedly, *CiFT3* correlated better to floral-inductive treatments (Nishikawa et al., 2009). Relative gene expression was calculated as a fold change ratio using Pfaffl's method (Pfaffl, 2001) with sliding-window efficiencies calculated for each reaction using the *slwin* function in the *qpcR* R package (Ritz and Spiess, 2008).

FLOWERING DATA. The number of inflorescences and new vegetative shoots was counted on the flush induced by the experimental treatments. Counts were made on all six- to seven-node-long shoots formed during the previous year present in each tree. The type of inflorescence (leaf-abundant, leaf-deficient, leafless, and single flowers) formed was also

registered. Flowering data are presented as the average number of inflorescences per shoot (six to seven nodes long) per tree.

STATISTICAL ANALYSIS. Both experiments were conducted separately using a completely randomized design with four single-tree replicates per treatment. Levels of gene expression were analyzed with analysis of variance using a repeated measurements model. Flowering data were analyzed using *t* tests. Mean fold change of transcript levels was transformed to a logarithmic scale (log2) for statistical analysis, but the untransformed data are presented in the graphs. Statistically significant differences were declared at $P \leq 0.05$. All statistical analyses were executed in R (R Development Core Team, 2011).

Results

FLOWERING RESPONSE INDUCED BY WATER DEFICIT TREATMENT AT FLORAL-INDUCTIVE AND NON-INDUCTIVE TEMPERATURES. In both experiments, trees that had been exposed to water deficit produced more inflorescences than well-irrigated trees (Tables 1 and 2). At 23 °C, very few inflorescences (0.2 per shoot) were induced in irrigated trees, whereas trees that were exposed to water deficit produced an average of 2.0 inflorescences per shoot (Table 1). Most of the inflorescences formed in trees under water deficit were of the leaf-abundant type (more leaves than flowers). In addition to inflorescences, trees that had been exposed to water deficit also produced an average of 1.1 vegetative shoots compared with no new vegetative buds formed in irrigated trees. The newly formed vegetative shoots sprouted at more basal positions along the shoot, whereas inflorescences were formed at more apical positions (data not shown). These data indicate that the water deficit treatment applied was effective at inducing flowering in the experimental trees.

At 12 °C, new inflorescences were induced in irrigated trees, but trees that had been exposed to water deficit produced almost twice as many inflorescences per shoot (Table 2). Leaf-abundant inflorescences were the most common type of

inflorescence formed. Trees under water deficit produced significantly ($P \leq 0.05$) more leaf-abundant inflorescences and single flowers than irrigated trees, whereas no differences between both treatments were detected for leaf-deficient, leafless inflorescences and new vegetative shoots. These data indicate that the combined effect of water deficit and cool temperature on floral induction was greater than the effect of cool temperature alone.

EFFECT OF WATER DEFICIT ON THE ACCUMULATION OF *CsFT*, *CsSLI*, *CsAPI*, AND *CsLFY* TRANSCRIPTS AT NON-INDUCTIVE TEMPERATURES (23 °C). By the end of the water deficit treatment, accumulation of *CsFT* transcripts in leaves of trees kept under water deficit had increased ≈ 22 -fold relative to initial levels (Fig. 1). Statistically significant ($P \leq 0.05$) differences in the level of accumulation of *CsFT* were detected by Day 20 after the beginning of the experiment. Over the duration of the experiment, the accumulation of *CsFT* transcripts increased constantly. However, when water deficit was interrupted by irrigation, transcript accumulation returned to initial and control levels and remained at this level on Day 10 after the transfer. These results indicate that the accumulation of *CsFT* transcripts was induced by water deficit and was proportional to the duration of the water deficit treatment. In addition, these results show that accumulation of *CsFT* transcript was only sustained above basal levels for as long as the trees remained under water deficit.

No differences in the level of accumulation of *CsSLI* transcripts in buds were detected between trees under water deficit and irrigated trees while trees were exposed to water deficit (Fig. 1). However, after the water deficit was interrupted, levels of *CsSLI* transcript accumulation increased 1.5-fold in the buds relative to the levels in the well-irrigated control trees previously exposed to water deficit. These results show that *CsSLI* expression was unaffected by water deficit but was promoted after reirrigation.

Accumulation of *CsAPI* and *CsLFY* transcripts in buds was reduced significantly by Day 20 and onward by water deficit

Table 1. Effect of water deficit applied for 60 d at 23 °C on bud sprouting and flowering of two- to three-year-old sweet orange cuttings.^z

Treatment	Inflorescences	Leaf-abundant inflorescences ^y	Leaf-deficient inflorescences ^y	Leafless inflorescences	Single flowers	Vegetative shoots	Flowers
[mean $\pm$ SE (no./shoot)]							
Irrigated	0.2 $\pm$ 0.09	0.11 $\pm$ 0.07	0.04 $\pm$ 0.04	0.02 $\pm$ 0.02	0.04 $\pm$ 0.03	0 $\pm$ 0	0.39 $\pm$ 0.19
Water deficit	2.04 $\pm$ 0.51	1.68 $\pm$ 0.48	0.04 $\pm$ 0.04	0.16 $\pm$ 0.09	0.16 $\pm$ 0.07	1.12 $\pm$ 0.23	3.24 $\pm$ 0.86
	*	*	NS	*	*	*	*

^zFour single-tree replicates were used with 11 to 23 shoots sampled on each tree (mean number of nodes per shoot = 6.16 $\pm$ 0.27).

^yLeaf-abundant inflorescences (leaf:flower ratio 1 or greater), leaf-deficient inflorescences (leaf:flower ratio less than 1).

*Values are significantly different between treatments using Student's *t* test ($P \leq 0.05$); NS = nonsignificant.

Table 2. Effect of water deficit applied for 40 d at 12 °C on bud sprouting and flowering of two- to three-year-old sweet orange cuttings.^z

Treatment	Inflorescences	Leaf-abundant inflorescences ^y	Leaf-deficient inflorescences ^y	Leafless inflorescences	Single flowers	Vegetative shoots	Flowers
[mean $\pm$ SE (no./shoot)]							
Irrigated	1.47 $\pm$ 0.08	1.08 $\pm$ 0.05	0.07 $\pm$ 0.03	0.25 $\pm$ 0.03	0.07 $\pm$ 0.04	0.55 $\pm$ 0.11	3.35 $\pm$ 0.12
Water deficit	2.73 $\pm$ 0.45	2.25 $\pm$ 0.36	0.05 $\pm$ 0.07	0.21 $\pm$ 0.09	0.21 $\pm$ 0.06	0.91 $\pm$ 0.31	4.24 $\pm$ 0.73
	*	*	NS	NS	*	NS	*

^zFour single-tree replicates were used with 11 to 23 shoots sampled on each tree (mean number of nodes per shoot = 6.77 $\pm$ 0.31). Irrigated trees were also held at 12 °C during the experiment.

^yLeaf-abundant inflorescences (leaf:flower ratio 1 or greater), leaf-deficient inflorescences (leaf:flower ratio less than 1).

*Values are significantly different between treatments using Student's *t* test ($P \leq 0.05$); NS = nonsignificant.

Fig. 1. Transcript accumulation of *CsFT*, *CsSL1*, *CsAPI*, and *CsLFY* in two- to three-year-old 'Washington Navel' sweet orange cuttings under water deficit at 23 °C. Transcript accumulation was monitored in leaves (*CsFT*) and buds (*CsSL1*, *CsAPI*, and *CsLFY*) of four-month-old or older shoots using quantitative reverse transcription–polymerase chain reaction. Moderate water deficit (–2 MPa midday stem water potential) was imposed by reducing irrigation frequency and volume. Water deficit was removed on Day 60 (gray line). Symbols represent the means of four single-tree replicates $\pm$ SE (six shoots/tree). Transcript accumulation is relative to the levels of *CsGAPDH* and to the level of each gene in "well-irrigated" trees on Day 0.

with levels of transcripts of these two genes ranging between 0.5- and 0.75-fold relative to the levels in the irrigated controls (Fig. 1). After the interruption of the water deficit treatment, the accumulation of transcripts of both genes increased $\approx$ 2-fold and returned to initial and control levels 10 d afterward. These results indicate that water deficit, when present, negatively regulates the accumulation of *CsAPI* and *CsLFY* transcripts and that interruption of the water deficit promoted it.

EFFECT OF WATER DEFICIT ON THE ACCUMULATION OF *CsFT*, *CsSL1*, *CsAPI*, AND *CsLFY* TRANSCRIPTS AT FLORAL INDUCTIVE TEMPERATURES (12 °C). Accumulation of *CsFT* transcripts in leaves increased steadily once trees were transferred to the growth room at 12 °C (Fig. 2). The increase in the accumulation of *CsFT* transcripts was greater in trees under water deficit than in irrigated trees. By the end of the treatments, levels of *CsFT* in trees under water deficit had increased $\approx$ 34-fold relative to initial levels, whereas in irrigated trees, the increase was $\approx$ 12-fold; 3 d after the trees were transferred back to the greenhouse and irrigation restored in the trees previously under water deficit, the levels of *CsFT* transcripts had returned to near initial levels. Increasing levels of *CsFT* transcripts in leaves of well-irrigated trees in response to cool temperatures were in agreement with previous reports in the literature (Nishikawa et al., 2007, 2009). These results, along with the results of the flowering response, suggest that the accumulation of *CsFT* transcripts was an indicator of the overall level of floral induction perceived by the trees regardless of the stimuli producing it.

Levels of *CsSL1* were initially reduced relative to initial levels both in irrigated trees and trees under water deficit (Fig. 2). In trees under water deficit, levels of *CsSL1* remained below initial levels ($\approx$ 0.75-fold) until trees were transferred back to the greenhouse and irrigation was restored; at this point, accumulation of *CsSL1* transcripts returned to pre-treatment levels. The latter observation contrasts with the small ($\approx$ 1.5-fold) but significant increase in the accumulation of *CsSL1* transcripts measured after reirrigation in the experiment at 23 °C. In irrigated trees, accumulation of *CsSL1* transcripts was initially reduced but later increased from Day 20 onward reaching $\approx$ 2-fold increase relative to initial levels by the end of the experiment; 2 d after transfer to the greenhouse, levels of *CsSL1* were still $\approx$ 2-fold higher than initial levels. These results along with results from the experiment at 23 °C indicate that between cool temperature and water deficit, only cool temperature induced the accumulation of *CsSL1* transcripts while the stimulus was present.

Levels of *CsAPI* and *CsLFY* transcript accumulation in buds were reduced to between 0.25- and 0.75-fold after transfer to the growth room at 12 °C in both irrigated trees and trees under water deficit (Fig. 2). The accumulation of transcripts of both genes remained below initial levels until the trees were transferred back to the greenhouse and water deficit was interrupted. After transfer

to the greenhouse and interruption of the water deficit, the accumulation of transcripts of both genes increased $\approx$ 4-fold in trees previously exposed to water deficit and $\approx$ 3-fold in the irrigated trees also previously held at 12 °C. A reduction of the accumulation of *CsAPI* and *CsLFY* transcripts while trees were under floral inductive conditions was in agreement with results from the experiment conducted at 23 °C. The increased accumulation of transcripts after floral induction was interrupted was also in agreement with the results of the experiment at 23 °C and reports in the literature (Pillitteri et al., 2004a). Higher accumulation of *CsAPI* and *CsLFY* transcripts in buds of trees that underwent water deficit than in buds of well-irrigated trees and flowering data indicate that the overall level of accumulation of *CsAPI* and *CsLFY* was also an indicator of the overall level of induction of the trees.

Discussion

In citrus, flowering is only induced by prolonged exposure to either cool (less than 20 °C) temperatures or water deficit (Cassin et al., 1969; Moss, 1969), and only two other factors, gibberellin applications and the presence of fruit, have well-demonstrated effects negatively regulating the intensity of floral induction perceived by the trees (Monselise et al., 1964; Moss, 1971). In recent years, the patterns of expression of putative citrus orthologs of arabidopsis flowering genes have been characterized in response to cool temperatures (Li et al., 2010; Nishikawa et al., 2007, 2009; Pillitteri et al., 2004a;

Fig. 2. Transcript accumulation of *CsFT*, *CsSL1*, *CsAPI*, and *CsLFY* in two- to three-year-old ‘Washington Navel’ sweet orange cuttings under water deficit at 12 °C. Transcript accumulation was monitored in leaves (*CsFT*) and buds (*CsSL1*, *CsAPI*, and *CsLFY*) of four-month-old or older shoots using quantitative reverse transcription–polymerase chain reaction. Moderate water deficit (–2 MPa midday stem water potential) was imposed by reducing irrigation frequency and volume starting 7 d before transfer from 23 to 12 °C (gray area). Water deficit was removed on Day 40 (darker gray line) and trees were transferred to a room at 23 °C and irrigated to saturation. Symbols represent the means of four single-tree replicates $\pm$ SE (six shoots/tree). Transcript accumulation is relative to the levels of *CsGAPDH* and to the level of each gene in “well-irrigated” trees on Day 0.

Zhang et al., 2009, 2011), gibberellins (Muñoz Fambuena et al., 2012a), and the presence of fruit (Muñoz Fambuena et al., 2011, 2012b). In this work, we investigated the pattern of expression (measured as transcript accumulation) of a set of four putative flowering genes (*CsFT*, *CsSL1*, *CsAPI*, and *CsLFY*) during water deficit in potted sweet orange trees.

Increased accumulation of *CsFT* transcripts during water deficit reported in Figure 1 indicates that, similar to cool temperatures (Nishikawa et al., 2007), water deficit also upregulated the expression of this gene. This increased accumulation of *CsFT* was correlated to more inflorescences being formed in trees under water deficit than in well-irrigated trees (Table 1). Thus, the level of accumulation of citrus *FT* ortholog transcripts seems to be sensitive to all four factors, cool temperature, gibberellin application, fruit load, and water deficit (Muñoz Fambuena et al., 2011, 2012a, 2012b; Nishikawa et al., 2007, 2009) known to have a clear effect on citrus flowering with consistent correlations with flowering response. In arabidopsis, *FT* is a key integrator of floral promoting/inhibiting signals from the photoperiod, vernalization, autonomous, and gibberellin pathways (Amasino, 2010; Turck et al., 2008), which suggests that the role of *FT* orthologs as integrators of endogenous and exogenous signals regulating flowering likely is conserved in citrus and arabidopsis.

Higher accumulation of *CsFT* transcripts (Fig. 2) and more inflorescences (Table 2) forming in trees exposed to water deficit than in well-irrigated trees at 12 °C indicate similar and

probably additive effects from these two variables, suggesting that the level of accumulation of *CsFT* transcripts could be an estimator of the level of induction perceived by the trees. Contrary to arabidopsis, in which the inflorescence develops from a single shoot meristem at the end of the rosette resulting in a rather discrete flowering response, in citrus and many other tree crops, inflorescences develop from many apical and axillary meristems resulting in a more continuous distribution of possible flowering responses (Valiente and Albrigo, 2004). In arabidopsis, the protein encoded by *FT* is a mobile signal synthesized in the phloem of leaves (An et al., 2004; Mathieu et al., 2007) and transported to the shoot apical meristem (Corbesier et al., 2007) where, along with *FD* (Abe et al., 2005), it activates the expression of floral meristem identity genes (Michaels et al., 2005; Wigge et al., 2005). Like in our results (Figs. 1 and 2), in arabidopsis, accumulation of *FT* transcripts increases as the plants are exposed for a longer time to a floral-inductive treatment and is sharply reduced after the inductive stimulus is removed (Corbesier et al., 2007). In arabidopsis, the main consequence of the overexpression of *FT* is early flowering (Kobayashi et al., 1999) measured typically as days until bolting or the number of rosette leaves emitted before bolting. In transgenic *Poncirus trifoliata*, a close relative of citrus, ectopic expression of the *Citrus unshiu FT* ortholog resulted in extreme early flowering [weeks vs. years

(Endo et al., 2005)]. Similarly, *Pyrus communis* and arabidopsis plants overexpressing *CiFT* also flowered earlier than untransformed plants (Kobayashi et al., 1999; Matsuda et al., 2009). In citrus, flowering intensity can be evaluated quantitatively and qualitatively and is related to the duration and intensity of floral inductive conditions (Moss, 1969; Southwick and Davenport, 1986). Quantitatively, the number of buds developing into inflorescences is proportional to the intensity of floral induction. Qualitatively, the leaf:flower ratio of inflorescences is inversely proportional to floral induction intensity (Moss, 1969). Our results indicate that the level of accumulation of *CsFT* could be a predictor to comparatively estimate flowering intensity in terms of the number of inflorescences induced. However, our results do not support a correlation among the level of accumulation of *CsFT* transcripts, intensity of floral induction, and the leaf:flower ratio of the inflorescences induced, because no differences were detected in the number of leaf-deficient or leafless inflorescences despite differences in the level of accumulation of *CsFT* transcripts. A shift toward leaf-deficient and leafless inflorescences would be expected at higher perceived levels of induction (Moss, 1969). In bud samples, the level of accumulation of *CsAPI* and *CsLFY* transcripts was correlated with the number of inflorescences induced and also with the level of accumulation of *CsFT* transcripts at the end of the inductive treatment. Because the bud samples in which *CsAPI* and *CsLFY* transcripts were quantified consisted of a pool of six buds, the increased

accumulation of *CsAPI* and *CsLFY* transcripts in induced buds could be interpreted as higher expression of the genes in individual buds or more buds activating the expression of these genes.

Reduced accumulation of *CsSLI* transcripts in trees under water deficit at any temperature but increased accumulation of *CsSLI* transcripts in well-irrigated trees at 12 °C (Figs. 1 and 2) indicates that water deficit acts as a negative regulator of the expression of this gene, whereas cool temperatures act as a promoter of its expression. In *Arabidopsis*, *SOC1* (*CsSLI* putative ortholog) integrates signals from the main four flowering pathways (Lee and Lee, 2010). *SOC1* can act downstream of *FT* or in a *FT*-independent manner in the shoot meristem to promote the expression of *LFY* in conjunction with *AGL24* (Lee et al., 2008). Thus, assuming a correlation between transcript accumulation and protein transport and activity of *CsFT*, the negative effect of water deficit on the accumulation of *CsSLI* could be a consequence of a water-deficit-specific signal integrated between *CsFT* and *CsSLI* or simply a *CsFT*-independent signal integrated at *CsSLI* to inhibit its expression.

Although water deficit reduced the accumulation of *CsSLI* transcripts during the treatments, the small increase in the accumulation of *CsSLI* transcripts after reirrigation (and transfer to 23 °C in the second experiment) suggests a carryover effect of water deficit on the expression of this gene. This response was also observed (even more clearly) in the accumulation of *CsAPI* and *CsLFY* transcripts in buds of trees previously exposed to water deficit (Figs. 1 and 2). In *Arabidopsis*, *API* and *LFY* act as floral meristem identity genes, and their activation triggers floral development (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Expression of *API* and *LFY* is activated by *FT* and *SOC1* and later reinforced by each other to maintain the floral identity of the meristem (Liljegren et al., 1999; Wagner et al., 1999; Wigge et al., 2005). Expression of *SOC1* not only was required for upregulation of *CsLFY*, but also for the activation of B and C floral organ identity genes during flower development (Liu et al., 2009). In *Arabidopsis*, consistent upregulation of *API* has been reported to occur ≈16 h after transfer to floral-inductive photoperiods marking floral determination (Hempel et al., 1997). Contrary to model species and most deciduous trees in which floral inductive and growth-promoting conditions occur simultaneously, in citrus, floral meristem differentiation and determination are only identifiable when growth-promoting conditions resume, which implies that floral-inductive conditions are no longer present or are less intense when growth starts (Lord and Eckard, 1985; Moss, 1976). Increased accumulation of *CsAPI* and *CsLFY* transcripts has been reported in citrus after floral induction by cool temperatures (Pillitteri et al., 2004a) providing molecular support for the hypothesis of floral differentiation occurring only after floral induction but not simultaneously in citrus. Our results using water deficit induction (Fig. 1) are also consistent with the latter observation.

In recent years, much interest has arisen over the conservation of the genetic framework regulating flowering among species. Although knowledge about the genetic-molecular mechanisms regulating flowering in model species has contributed significantly to the identification of potential regulators of flowering in crop species, evolution and adaptation to different environments have likely resulted in important modifications to these mechanisms. In citrus, floral induction is practically photoperiod-insensitive, gibberellin treatments induce responses opposite to those induced in *Arabidopsis*, and the role

of cool temperatures is not comparable to the flowering-enabling role of vernalization. Our results and those of others (Muñoz Fambuena et al., 2011, 2012a; Nishikawa et al., 2007; Pillitteri et al., 2004a) support the involvement of *CsFT* as an integrator of signals regulating flowering in citrus and probably a defining factor for the number of inflorescences to be formed after induction as well as the likely conservation of *CsAPI* and *CsLFY* function as floral meristem identity genes. Nonetheless, the characterization of the genetic framework upstream of these genes regulating their expression remains mostly unexplored in citrus. Determining the composition and topology of the network regulating flowering in citrus will be useful to understand how citrus and other species perceive developmental signals from the environment and to engineer mechanisms to manipulate the reproductive biology of these species for horticultural purposes.

Literature Cited

- Abbot, C.E. 1935. Blossom-bud differentiation in citrus trees. *Amer. J. Bot.* 22:476–485.
- Abe, M., Y. Kobayashi, S. Yamamoto, Y. Daimon, A. Yamaguchi, Y. Ikeda, H. Ichinoki, M. Notaguchi, K. Goto, and T. Araki. 2005. FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science* 309:1052–1056.
- Albrigo, L.G. and V. Galen-Saúco. 2004. Flower bud induction, flowering and fruit-set of some tropical and subtropical fruit tree crops with special reference to citrus. *Acta Hort.* 632:81–91.
- Albrigo, L.G., J.I. Valiente, and C. Van Parys de Wit. 2006. Influence of winter and spring weather on year-to-year citrus fruit set and yield variation in São Paulo, Brazil. *Proc. Intl. Soc. Citricult.* 1:263–267.
- Amasino, R. 2010. Seasonal and developmental timing of flowering. *Plant J.* 61:1001–1013.
- An, H., C. Roussot, P. Suarez-Lopez, L. Corbesier, C. Vincent, M. Pineiro, S. Hepworth, A. Mouradov, S. Justin, C. Turnbull, and G. Coupland. 2004. CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of *Arabidopsis*. *Development* 131:3615–3626.
- Balasubramanian, S., S. Sureshkumar, J. Lempe, and D. Weigel. 2006. Potent induction of *Arabidopsis thaliana* flowering by elevated growth temperature. *PLoS Genet.* 2:e106.
- Blázquez, M.A., J.H. Ahn, and D. Weigel. 2003. A thermosensory pathway controlling flowering time in *Arabidopsis thaliana*. *Nat. Genet.* 33:168–171.
- Blázquez, M.A., R. Green, O. Nilsson, M.R. Sussman, and D. Weigel. 1998. Gibberellins promote flowering of *Arabidopsis* by activating the *Leafy* promoter. *Plant Cell* 10:791–800.
- Cassin, J., J. Bourdeuet, A. Fougue, V. Furon, J.P. Gaillard, J. Bourdelles, G. Montaguad, and C. Moreuil. 1969. The influence of climate upon the blooming of citrus in tropical areas. *Proc. First Intl. Citrus Symp.* 1:315–323.
- Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156–159.
- 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.
- 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.
- Furr, J.R., W.C. Cooper, and P.C. Reece. 1947. An investigation of flower formation in adult and juvenile citrus trees. *Amer. J. Bot.* 34:1–8.
- García-Luís, A. and M. Kandušer. 1995. Changes in dormancy and sensitivity to vernalization in axillary buds of Satsuma mandarin

- examined in vitro during the annual cycle. *Ann. Bot. (Lond.)* 76:451–455.
- García-Luis, A.P., P. Santamarina, and J.L. Guardiola. 1989. Flower formation from *Citrus unshiu* buds cultured in vitro. *Ann. Bot. (Lond.)* 64:515–519.
- Hempel, F., D. Weigel, M. Mandel, G. Ditta, P. Zambryski, L. Feldman, and M. Yanofsky. 1997. Floral determination and expression of floral regulatory genes in *Arabidopsis*. *Development* 124:3845–3853.
- Kim, D.-H., M.R. Doyle, S. Sung, and R.M. Amasino. 2009. Vernalization: Winter and the timing of flowering in plants. *Annu. Rev. Cell Dev. Biol.* 25:277–299.
- Kobayashi, Y., H. Kaya, K. Goto, M. Iwabuchi, and T. Araki. 1999. A pair of related genes with antagonistic roles in mediating flowering signals. *Science* 286:1960–1962.
- Lee, J. and I. Lee. 2010. Regulation and function of SOC1, a flowering pathway integrator. *J. Expt. Bot.* 61:2247–2254.
- Lee, J., M. Oh, H. Park, and I. Lee. 2008. SOC1 translocated to the nucleus by interaction with AGL24 directly regulates *Leafy*. *Plant J.* 55:832–843.
- Li, Z.-M., J.-Z. Zhang, L. Mei, X.-X. Deng, C.-G. Hu, and J.-L. Yao. 2010. *PtSVP*, an *SVP* homolog from trifoliate orange (*Poncirus trifoliata* L. Raf.), shows seasonal periodicity of meristem determination and affects flower development in transgenic *Arabidopsis* and tobacco plants. *Plant Mol. Biol.* 74:129–142.
- Liljegen, S.J., C. Gustafson-Brown, A. Pinyopich, G.S. Ditta, and M.F. Yanofsky. 1999. Interactions among *Apetala*, *Leafy*, and *Terminal Flower1* specify meristem fate. *Plant Cell* 11:1007–1018.
- Liu, C., W. Xi, L. Shen, C. Tan, and H. Yu. 2009. Regulation of floral patterning by flowering time genes. *Dev. Cell* 16:711–722.
- Lord, E.M. and K.J. Eckard. 1985. Shoot development in *Citrus sinensis* L. (Washington Navel orange). I. Floral and inflorescence ontogeny. *Bot. Gaz.* 146:320–326.
- Mandel, M.A. and M. Yanofsky. 1995. A gene triggering flower formation in *Arabidopsis*. *Nature* 377:522–524.
- Mathieu, J., N. Warthmann, F. Küttner, and M. Schmid. 2007. Export of FT protein from phloem companion cells is sufficient for floral induction in *Arabidopsis*. *Curr. Biol.* 17:1055–1060.
- Matsuda, N., K. Ikeda, M. Kurosaka, T. Takashina, K. Isuzugawa, T. Endo, and M. Omura. 2009. Early flowering phenotype in transgenic pears (*Pyrus communis* L.) expressing the *CiFT* gene. *J. Jpn. Soc. Hort. Sci.* 78:410–416.
- McCutchan, H. and K. Shackel. 1992. Stem water potential as a sensitive indicator of water stress in prune trees (*Prunus domestica* L. cv. French). *J. Amer. Soc. Hort. Sci.* 117:607–611.
- Michaels, S.D., E. Himelblau, S.Y. Kim, F.M. Schomburg, and R.M. Amasino. 2005. Integration of flowering signals in winter-annual *Arabidopsis*. *Plant Physiol.* 137:149–156.
- Monselise, S.P., R. Goren, and A. Halevy. 1964. Chemical inhibition and promotion of citrus flower bud induction. *Proc. Amer. Soc. Hort. Sci.* 84:141–146.
- Moss, G.I. 1969. Influence of temperature and photoperiod on flower induction and inflorescence development in sweet orange (*Citrus sinensis* L. Osbeck). *J. Hort. Res.* 44:311–320.
- Moss, G.I. 1971. Effect of fruit on flowering in relation to biennial bearing in sweet orange (*Citrus sinensis*). *J. Hort. Sci.* 46:177–184.
- Moss, G.I. 1976. Temperature effects on flower initiation in sweet orange (*Citrus sinensis*). *Austral. J. Agr. Res.* 27:399–407.
- Muñoz Fambuena, N., C. Mesejo, M. Carmen González-Mas, E. Primo-Millo, M. Agustí, and D.J. Iglesias. 2011. Fruit regulates seasonal expression of flowering genes in alternate-bearing moncada mandarin. *Ann. Bot. (Lond.)* 108:511–519.
- Muñoz Fambuena, N., C. Mesejo, M. González-Mas, D. Iglesias, E. Primo-Millo, and M. Agustí. 2012a. Gibberellic acid reduces flowering intensity in sweet orange [*Citrus sinensis* (L.) Osbeck] by repressing *CiFT* gene expression. *J. Plant Growth Regul.* 31:529–536.
- Muñoz Fambuena, N., C. Mesejo, M.C. González-Mas, E. Primo-Millo, M. Agustí, and D.J. Iglesias. 2012b. Fruit load modulates flowering-related gene expression in buds of alternate-bearing Moncada mandarin. *Ann. Bot. (Lond.)* 110:1109–1118.
- 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.
- 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. Expt. Bot.* 58:3915–3927.
- Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45.
- Pillitteri, L.J., C.J. Lovatt, and L.L. Walling. 2004a. Isolation and characterization of a *Terminal Flower* homolog and its correlation with juvenility in citrus. *Plant Physiol.* 135:1540–1551.
- Pillitteri, L.J., C.J. Lovatt, and L.L. Walling. 2004b. Isolation and characterization of *Leafy* and *Apetalal* homologues from *Citrus sinensis* L. Osbeck ‘Washington’. *J. Amer. Soc. Hort. Sci.* 129:846–856.
- R Development Core Team. 2011. R: A language and environment for statistical computing. 3 Jan. 2013. <<http://www.r-project.org/>>.
- Ritz, C. and A.N. Spiess. 2008. qpcR: An R package for sigmoidal model selection in quantitative real-time polymerase chain reaction analysis. *Bioinformatics* 24:1549–1551.
- Samach, A., H. Onouchi, S.E. Gold, G.S. Ditta, Z. Schwarz-Sommer, M.F. Yanofsky, and G. Coupland. 2000. Distinct roles of Constans target genes in reproductive development of *Arabidopsis*. *Science* 288:1613–1616.
- Sambrook, J., E. Fritsch, and T. Maniatis. 1989. Molecular cloning, A laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, New York, NY.
- Sauer, M.R. 1954. Flowering in the sweet orange. *Austral. J. Agr. Res.* 5:649–657.
- Scholander, P.F., A.D. Hammel, A.D. Bradstreet, and E.A. Hemmingsen. 1965. Sap pressure in vascular plants. *Science* 148:339–346.
- Southwick, S.M. and T.L. Davenport. 1986. Characterization of water stress and low temperature effects on flower induction in citrus. *Plant Physiol.* 81:26–29.
- Tan, F.-C. and S.M. Swain. 2007. Functional characterization of *AP3*, *SOC1* and *WUS* homologues from citrus (*Citrus sinensis*). *Physiol. Plant.* 131:481–495.
- Turck, F., F. Fornara, and G. Coupland. 2008. Regulation and identity of florigen: Flowering locus t moves center stage. *Annu. Rev. Plant Biol.* 59:573–594.
- Valiente, J.I. and L.G. Albrigo. 2004. Flower bud induction of sweet orange trees [*Citrus sinensis* (L.) Osbeck]: Effect of temperature, crop load and bud age. *J. Amer. Soc. Hort. Sci.* 129:158–164.
- Wagner, D., R.W.M. Sablowski, and E.M. Meyerowitz. 1999. Transcriptional activation of *APETALA1* by *LEAFY*. *Science* 285:582–584.
- Weigel, D. and O. Nilsson. 1995. A developmental switch sufficient for flower initiation in diverse plants. *Nature* 377:495–500.
- Wigge, P.A., M.C. Kim, K.E. Jaeger, W. Busch, M. Schmid, J.U. Lohmann, and D. Weigel. 2005. Integration of spatial and temporal information during floral induction in *Arabidopsis*. *Science* 309:1056–1059.
- Zhang, J.-Z., X.-Y. Ai, L.-M. Sun, D.-L. Zhang, W.-W. Guo, X.-X. Deng, and C.-G. Hu. 2011. Molecular cloning and functional characterization of genes associated with flowering in citrus using an early-flowering trifoliate orange (*Poncirus trifoliata* L. Raf.) mutant. *Plant Mol. Biol.* 76:187–204.
- Zhang, J.-Z., Z.-M. Li, L. Mei, J.-L. Yao, and C.-G. Hu. 2009. *PtFLC* homolog from trifoliate orange (*Poncirus trifoliata*) is regulated by alternative splicing and experiences seasonal fluctuation in expression level. *Planta* 229:847–859.