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Gibberellic Acid Reduces Flowering Intensity in Sweet Orange [*Citrus sinensis* (L.) Osbeck] by Repressing *CiFT* Gene Expression

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Abstract In *Citrus*, gibberellic acid (GA_3) applied at the floral bud inductive period significantly reduces flowering intensity. This effect is being used to improve the fruit set of parthenocarpic cultivars that tend to flower profusely. However, the molecular mechanisms involved in the process remain unclear. To contribute to the knowledge of this phenomenon, adult trees of 'Salustiana' sweet orange were sprayed at the floral bud inductive period with 40 mg L^{-1} of GA_3 and the expression pattern of flowering genes was examined up to the onset of bud sprouting. Trees sprayed with paclobutrazol (PBZ, 2,000 mg L^{-1}), a gibberellin biosynthesis inhibitor, were used to confirm the effects, and untreated trees served as control. Bud sprouting, flowering intensity, and developed shoots were evaluated in the spring. GA_3 significantly reduced the number of flowers per 100 nodes by 72% compared to the control, whereas PBZ increased the number by 123%. Data of the expression pattern of flowering genes in leaves of GA_3-treated trees revealed that this plant growth regulator inhibited flowering by repressing relative expression of the homolog of *FLOWERING LOCUS T*, *CiFT*, whereas PBZ increased flowering by boosting its expression. The activity of the homologs *TERMINAL FLOWER 1*, *FLOWERING LOCUS C*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*, and *APETALA1* was not affected by the treatments. The number of flowers per inflorescence, in both leafy and leafless inflorescences, was not altered by GA_3 but increased with PBZ; the latter paralleled *LEAFY* relative expression. These results suggest that GA_3 inhibits flowering in *Citrus* by repressing *CiFT* expression in leaves.

Keywords *AP1* · *Citrus* · *FLC* · Flowering · *FT* · Gene expression · Gibberellic acid · *LFY* · *SOC1* · *TFL1*

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Introduction

Genetic and molecular approaches have been used to identify genes that regulate flower initiation and development in *Arabidopsis* and other model annual plants. The discovery of similar genes in other species has facilitated research into the control of flowering in a wide range of annual, biennial, and perennial plants. Thus, flowering genes have been isolated from major woody fruit tree species such as apple (Kotoda and others 2000; Sung and others 1999, 2000); grape (Boss and others 2003, 2006); pear, quince, and loquat (Esumi and others 2005); and peach (Zhang and others 2008), indicating that they are conserved during the evolution of flowering plants.

In *Citrus*, orthologs to *FLOWERING LOCUS TIME* (*FT*) (Endo and others 2005), *TERMINAL FLOWER 1* (*TFL1*) (Pillitteri and others 2004b), *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Peña and others 2001), *FLOWERING LOCUS C* (*FLC*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) (Muñoz-Fambuena and others 2011) have now been isolated and characterized. The isolation of *FT* and its ectopic expression conferring early flowering in *Poncirus trifoliata* (Endo and others 2005) and its repression by fruit load in 'Moncada' hybrid mandarin [Clementine 'Oroval' (*Citrus clementina* Hort ex Tanaka) × 'Kara' mandarin (*C. unshiu* Marc. × *C. nobilis*

Lou.)](Muñoz-Fambuena and others 2011) suggest its pivotal role in inducing *Citrus* flowering.

Many *Citrus* cultivars tend to alternate bearing. Heavy fruit load accentuates biennial bearing by reducing flower production (Moss 1971), with the effect depending on the length of time the fruit remains on the tree (Martínez-Fuentes and others 2010). There is evidence that the inhibitory effect of fruit load on flowering is due to gibberellin (GA) export from the fruit (Luckwill 1970), because reduced levels of endogenous GA correlated with flower initiation (Koshita and others 1999) and GA biosynthesis inhibitors enhanced flowering (Monselise and others 1966; Harty and van Staden 1988), although with some restrictions such as crop load, tree developmental stage, orchard practices, and varietal characteristics (Martínez-Fuentes and others 2004). Moreover, applying GA_3 may suppress floral initiation and markedly reduce the number of developing shoots (Monselise and Halevy 1964; Guardiola and others 1982), with the sensitivity of buds coinciding in time with the greater effect of fruit-inhibiting flowering (Martínez-Fuentes and others 2010). It has also been reported that GA promotes vegetative growth in perennials at the expense of reproductive development (Boss and Thomas 2002), as occurs in *Citrus* (Guardiola and others 1982; García-Luis and others 1986; Martínez-Fuentes and others 2010).

Despite this evidence, it is not yet clear whether GA has a true physiological role in the regulation of flowering in *Citrus*. In addition, there is limited information on the molecular mechanisms involved in its effect on inhibiting flower initiation (see review by Mutasa-Göttgens and Hedden 2009). This is, in part, because in many annual plants, such as *Arabidopsis*, flowering is completely dependent on GA signaling (Wilson and others 1992), leading to the transition from the vegetative meristem to the inflorescence meristem at the shoot apex.

The aim of this study was to determine the effect of GA on the expression of putative ortholog genes involved in flowering pathways of sweet orange to provide insight into the molecular mechanisms underlying GA inhibition of flowering in *Citrus*. The effect was determined by applying GA_3 at the floral bud inductive period and using paclobutrazol, a GA biosynthesis inhibitor, to confirm the effect. We used 'Salustiana' sweet orange, a 'Comuna' sweet orange bud mutation that ripens late in February, is harvested from March to May, and has some tendency to alternate bearing.

Materials and Methods

Plant Material and Treatments

The experiment was carried out using 15-year-old 'Salustiana' sweet orange fully productive trees [*Citrus sinensis* (L.) Osbeck)], grafted onto Carrizo citrange rootstock [*Poncirus trifoliata* (L.) Raf. × *C. sinensis* (L.) Osbeck], located in a commercial orchard in Museros (Valencia, Spain). The trees were planted 6 m × 5 m apart, in a loamy clay soil, with drip irrigation. Due to the inhibitory effect of fruit on flowering, only trees bearing moderate fruit load ($\sim$80 kg tree^{-1}) were selected. In early December, 40 mg L^{-1} of GA_3 (Arabelex-L; 1.6% w/v; Aragro, Madrid, Spain) and 2,000 mg L^{-1} of PBZ (Cultar; 25% w/v; Syngenta Agro, S.A., Madrid, Spain) were sprayed onto entire trees with a hand-gun sprayer at a pressure of 25–30 atm, wetting the tree to the point of run-off and using 10 L per tree. A nonionic wetting agent (alkyl polyglycol ether, 20% w/v) was added at a rate of 0.05% v/v. Untreated trees served as control.

Flowering Evaluation

At the treatment date, four branches per tree, similar in size (2–3-cm base diameter), were chosen from around the tree at 1.5–2 m above the soil level and totaling some 2,000 nodes and were labeled for flowering evaluation in the following spring. Prior to anthesis, the initiated shoots as well as their flowers and leaves were counted and were classified according to Guardiola and others (1977). Unsprouted nodes were also counted. Calculations were made based on the number of nodes per branch, the number of developed shoots per branch, the number of flowers per shoot, the number of shoots per 100 nodes, and the number of flowers per 100 nodes. The results were expressed per 100 nodes to compensate for the differences in the size of the branches selected for counting. Only buds younger than 24 months of age were considered for the counts because older buds seldom contribute to the spring flush.

RNA Extraction and RT-PCR

From the treatment date (11 December) to the onset of bud sprouting (late February) 30 fully developed, autumn flush (that is, nonbearing shoots), mature adult leaves per tree from control and GA_3- and PBZ-treated trees were randomly collected for RNA extraction. Samples were ground and stored at –80°C for RNA extraction. Six trees were used for the extractions.

Total RNA was isolated from frozen tissue using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). RNA samples were treated with RNase free DNase (Qiagen) through column purification following the manufacturer's instructions. RNA quality was tested by the OD_{260}/OD_{280} ratio and gel electrophoresis. RNA concentration was determined by fluorometric assays with the RiboGreen dye (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. Three fluorometric assays per RNA sample were performed. Quantitative real-time

RT-PCR was performed with a LightCycler 2.0 instrument (Roche Diagnostic, Basel, Switzerland) equipped with LightCycler software ver. 4.0. One-step RT-PCR was carried out. Reactions contained 2.5 U of MultiScribe Reverse Transcriptase (Applied Biosystems, Carlsbad, CA, USA), 1 U of RNase inhibitor (Applied Biosystems), 2 mL LC FastStart DNA MasterPLUS SYBR® Green I (Roche Diagnostic), 25 ng total RNA and 250 nM of the specific forward and reverse primers of each gene in a total volume of 10 mL. Incubations were carried out at 48°C for 30 min, 95°C for 10 min, followed by 45 cycles at 95°C for 2 s, 58°C for 8 s, and 72°C for 8 s. Fluorescent intensity data were acquired during the 72°C extension step and transformed into relative mRNA values using a tenfold dilution series of RNA sample as standard curve. Relative mRNA levels were then normalized to total mRNA amounts (Bustin 2002; Hashimoto and others 2004), and an expression value of 1 was arbitrarily assigned to the first sample of the control trees in each case. *β-Actin* was used as the reference gene, according to Yan and others (2011). Specificity of the amplification reactions was assessed by post-amplification dissociation curves and by sequencing the reaction product.

Putative genes were identified through homology search with related genes from an EST database of a random 5′ Clemenules mandarin (*C. clementina* Hort ex Tan.) full-length cDNA library (Terol and others 2008). Synthetic oligonucleotides were designed to amplify the gene of the selected clones and, as stated before, sequenced for confirmation. Details about the forward and reverse primers are given in Table 1.

Statistical Analyses

The experiment was laid out as a randomized complete block design, with single-tree plots and six replicates per treatment. Analysis of variance was performed on the data, and means were separated using Duncan's new multiple-range test. Percentages were transformed to arcsin to homogenize the variance. The program Statgraphics Plus for Windows ver. 5.1 (Statistical Graphics, Englewood Cliffs, NJ, USA) was used.

Results

GA_3—Flowering Relationship

Application of 40 mg L^{-1} of GA_3 at the flower bud inductive period (winter rest period for *Citrus*) significantly reduced the number of flowers per 100 nodes of 'Salustiana' sweet orange by 72% ($p \leq 0.01$) in comparison with control trees (Table 2). This treatment also reduced bud sprouting by 40% ($p \leq 0.05$) compared to the control. Leafless single-flowered shoots and leafless inflorescences were reduced on average from 3.6 to 1.3 and from 5.1 to 1.9 per 100 nodes, respectively, due to treatment, with differences being statistically significant ($p \leq 0.05$). Among flowered leafy shoots, only inflorescences were significantly reduced by GA_3 from 5.6 to 1.2 per 100 nodes ($p \leq 0.05$). Conversely, GA_3 significantly increased vegetative shoots from 3.8 to 9.0 ($p \leq 0.05$) (Table 2).

PBZ applied at a concentration of 2,000 mg L^{-1} yielded an opposite trend. The number of flowers per 100 nodes and percentage of sprouted buds were increased by 123% and 74%, respectively, compared to the control ($p \leq 0.05$) (Table 2). For leafy shoots, both single-flowered and inflorescences were not significantly altered by this treatment. However, for the leafless shoots, both single-flowered and inflorescences were significantly increased from 3.6 to 8.8 ($p \leq 0.05$) and from 5.1 to 16.3 shoots per 100

Table 1 List of primers used for quantitative real-time PCR

Annotation	EST code[a]	5′-Direct primer-3′ 5′-Reverse primer-3′	Predicted product (bp)
CiFT	aCL6275Contig1	GGGAGGCAGACTGTTTATGC	84
		CGGAGGTCCCAGATTGTAAA	
SOC1	aCL2263Contig1	CCTCGTTCAACCGTTACCAT	100
		GCAAGCCTTCTCTTGCTTTG	
FLC	aCL8484Contig1	CGCGACAAACAGAGTGAAAA	110
		TGTCTCGCAATCTCCTGTTG	
CsTFL	aCL6873Contig1	TCCGTCCACAGTTGTTTCAA	105
		TCACTAGGGCCAGGAACATC	
CsLFY	aC34107C06EF_c	TCTTGATCCAGGTCCAGAACATC	63
		TAGTCACCTTGGTTGGGCATT	
CsAP1	aCL9055Contig1	CAAAACCAGGTTCCCAACAC	139
		ACGAACATACGGGTTCAAGG	

[a] EST code refers to the database entry available in Citrus Functional Genomics Project (CFGP; http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/)

Table 2 Effect of GA_3 (40 mg L^{-1}) and PBZ (2,000 mg L^{-1}) applied to entire trees during the floral bud inductive period (11 December) on bud sprouting and flowering of 'Salustiana' sweet orange trees

	Control	GA_3	PBZ
Flowers	47.2 ± 3.1 b	13.5 ± 1.7 a	104.5 ± 9.2 c
Sprouted buds[a]	23.0 ± 1.9 b	13.8 ± 1.3 a	39.7 ± 2.8 c
Leafless shoots[b]			
Single flowered	3.6 ± 0.7 b	1.3 ± 0.5 a	8.8 ± 1.3 c
Inflorescence	5.1 ± 1.2 b	1.9 ± 0.2 a	16.3 ± 2.9 c
Leafy shoots			
Single flowered	0.8 ± 0.1 a	1.0 ± 0.2 a	0.9 ± 0.2 a
Inflorescence	5.6 ± 0.8 b	1.2 ± 0.3 a	5.7 ± 1.1 b
Vegetative shoots	3.8 ± 0.4 b	9.0 ± 0.5 c	0.8 ± 0.1 a
No. flowers inflorescence^{-1}			
Leafy inflorescence	3.9 ± 0.3 a	3.4 ± 0.5 a	4.9 ± 0.1 b
Leafless inflorescence	3.7 ± 0.1 a	3.4 ± 0.3 a	4.2 ± 0.1 b

Each value is the mean of six trees ± SE. Different letters in the same line indicate significant differences ($p \leq 0.05$)

[a] Sprouted buds expressed as percent of total buds

[b] Number of shoots and flowers expressed per 100 nodes

nodes ($p \leq 0.01$), respectively. PBZ significantly reduced the number of vegetative shoots per 100 nodes (0.8) compared to the control (3.8; $p \leq 0.05$) (Table 2).

Interestingly, the number of flowers per shoot of both leafy and leafless inflorescences were not significantly altered by GA_3 in comparison with the control, with 3.9 and 3.4 flowers per leafy inflorescence and 3.7 and 3.4 flowers per leafless inflorescence, respectively, whereas PBZ increased flower number significantly up to 4.9 and 4.2 flowers per shoot for leafy and leafless inflorescences, respectively ($p \leq 0.05$). Neither GA_3 nor PBZ changed the number of leaves per shoot in any case, even that of vegetative shoots (data not shown).

Expression of Flowering-related Genes

The time course of the relative expression of *CiFT* in leaves throughout the study was significantly affected by GA_3 (Fig. 1a). Significant differences in mRNA transcripts between GA_3-treated trees and control trees were detected from 8 days after treatment (DAT) onward. The expression in control tree leaves increased progressively up to 32 DAT (mid-January), decreasing thereafter down to almost the initial value (Fig. 1a). Gene expression in leaves of GA_3-treated trees paralleled that of control trees but was significantly reduced by 16% on average, except for 80 DAT (late February) when no significant differences were found between control and treated trees (Fig. 1a). On the other hand, PBZ-treated trees significantly boosted the relative expression of *CiFT* in leaves by 30% on average from 8 DAT up to the end of February, which is the onset of bud sprouting. In this case, leaf gene expression also paralleled that of control trees, but with significantly higher values throughout the entire period studied (Fig. 1a).

Relative expression of *SOC1* in leaves was not significantly altered by treatments (Fig. 1b). At 8 DAT, activity was reduced by 50% on average compared to that at the date of treatment, remaining almost constant up to the end of January (50 DAT), with the increase afterward coinciding with the onset of bud sprouting. These changes throughout the period of the study were independent of treatments (Fig. 1b).

Figure 1c shows the time course of the relative expression of the *FLC* gene in leaves from control and GA_3- and PBZ-treated trees. From early December to the onset of bud break, no differences in gene expression were found. Activity in leaves remained almost stationary between 0.70 and 1.03, regardless of the treatment.

Relative expression of *CsTFL1* in leaves slightly decreased up to the middle of January (32 DAT), increasing twofold afterward up to the end of January (50 DAT); from then up to the onset of bud sprouting, the relative expression of *CsTFL1* decreased again down to the initial value (Fig. 1d). No GA_3 or PBZ effect was observed throughout the experiment.

In control and GA_3-treated trees, the relative expression of *CsLFY* in leaves did not show significant differences, remaining almost constant between 0.66 and 1.21 throughout the study (Fig. 1e). However, in PBZ-treated trees there was no treatment effect until 50 DAT (late in January) when mRNA transcripts in leaf significantly increased 1.8-fold (1.94) compared to control (1.12). In spite of the subsequent decline, a significantly higher relative expression of *CsLFY* in comparison to control and GA_3-treated trees was recorded in PBZ-treated leaves at bud break (Fig. 1e).

Relative expression of *CsAP1* in leaves decreased slightly up to the end of January, increasing thereafter and reaching almost the initial value at bud sprouting (Fig. 1f). Activity in GA_3- and PBZ-treated trees paralleled that of control trees, with no significant treatment effect (Fig. 1f).

Discussion

The role of GA in inhibiting floral initiation in woody perennials has been extensively reported (see review by Wilkie and others 2008). Applied GA_3 inhibits flowering in *Citrus* (Monselise and Halevy 1964), avocado (Salazar-García and Lovatt 1998), peach (García-Pallás and Blanco 2001), plum (González-Rossia and others 2006), sweet

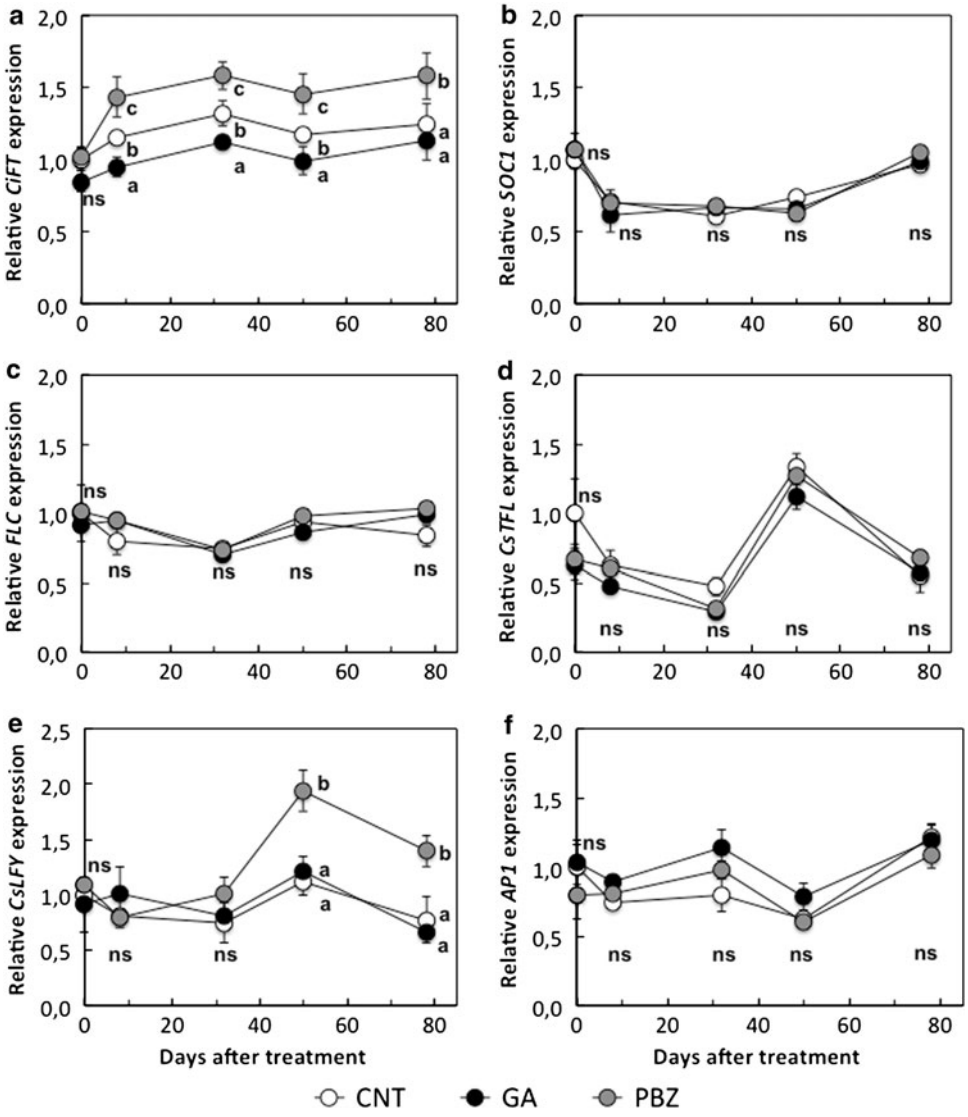

Fig. 1 Effect of gibberellic acid (GA, 40 mg L^{-1}) and paclobutrazol (PBZ, 2,000 mg L^{-1}) applied at the floral bud inductive period on the time course of *CiFT* (**a**), *SOC1* (**b**), *FLC* (**c**), *CsTFL* (**d**), *CsLFY* (**e**), and *CsAP1* (**f**) relative expression up to bud sprouting in the leaves of 'Salustiana' sweet orange. Data are the mean ± SE of four replicates. Different letters for the same sampling date indicate significant difference ($p \leq 0.05$); *ns* not significant; date of treatment, 11 December

cherry (Lenahan and others 2006), and loquat (Reig and others 2011). Up to now, it has been suggested that GA acts indirectly on the floral process by delaying bud formation (Bertelsen and others 2002), reducing bud sprouting (Guardiola and others 1982), and promoting vegetative growth at the expense of reproductive development (Boss and Thomas 2002). Guardiola and others (1982) suggested that some stimulus coming from the leaves allows the expression of the floral bud potential, with the application of GA_3 interfering with it. Later, García-Luis and others (1986) demonstrated that GA_3 acts directly on the bud. Despite this, the molecular signaling pathways involved in the floral inhibition by GA and the sites of GA biosynthesis and action within flowers in *Citrus* are unknown.

There is some controversy about whether the buds could sense the floral stimulus that induces flowering in the absence of leaves (Davenport 2000; Nishikawa and others 2007), but the results demonstrate that leaves are necessary for flowering in woody perennials such as mango (Singh 1959), loquat (Fatta del Bosco 1961), olive (Hartmann and others 1967), *Citrus* (Ayalon and Monselise 1960), and lychee (Ying and Davenport 2004). Moreover, in *Citrus* low temperatures induce the expression of *CiFT* in leaves (Nishikawa and others 2007, 2009). This expression is repressed by fruit load (Muñoz-Fambuena and others 2011) during the floral bud inductive period. To this effect, leaves from GA_3-treated trees (low return bloom during forthcoming spring) showed significantly decreased mRNA transcript levels compared to those from control trees (high return bloom). Moreover, they showed significantly increased relative expression of *CiFT* due to PBZ compared to control trees. Accordingly, our results shed light on the relationship between GA_3 application, the expression of *CiFT* in leaves, and flowering, providing new evidence of an eventual direct role for *FT* in the control of flowering regulated by GA levels.

In 'Washington' navel sweet orange adult trees, *CsTFL1* transcript accumulation was not detected in vegetative tissues (Pillitteri and others 2004b), in contrast to our results on 'Salustiana' sweet orange and those on Satsuma mandarin, the hybrid 'Moncada' mandarin, and *Poncirus trifoliata* (Nishikawa and others 2007, 2009; Muñoz-Fambuena and others 2011) in which *CsTFL1* is expressed in leaves. In addition, transcripts of *CsTFL1* have been detected only in developing vegetative shoots, suggesting that *CsTFL1* may affect the vegetative growth in adult *Citrus* trees (Nishikawa and others 2007). In the hybrid 'Moncada' mandarin no differences between *on* and *off* trees regarding the relative expression of *CsTFL1* in leaves were detected (Muñoz-Fambuena and others 2011). Our data agree with these results and show no differences in *CsTFL1* expression with either GA_3 or PBZ treatment, indicating that the effect of GA_3 inhibiting flowering in *Citrus* is not dependent on this gene.

There is very little information about the effect of GA on regulation of *FLC* gene expression. Increases in the relative expression of *FLC* measured in *Citrus* leaves at the floral bud inductive period have been related to the suppression of *CiFT* activity due to fruit load and, thus, to the inhibition of the floral process (Muñoz-Fambuena and others 2011). This agrees with the hypothesis that *FLC* directly repressed the expression of *FT* as well as that of *SOC1* in *Arabidopsis* (Searle and others 2006). Our results, however, show that GA_3, which inhibits flowering when applied at the floral bud inductive period, did not modify the relative expression of *FLC* or *SOC1*, in contrast with fruit load inhibiting flowering which exhibits a repressive effect of *SOC1* expression in leaves (Muñoz-Fambuena and others 2011). Moreover, PBZ, an inhibitor of GA biosynthesis that promotes flowering, does not modify the relative expression of *FLC* or *SCO1*. These results suggest that complementary gene expression pathways might be involved in floral bud inductive processes. Zhang and others (2009) discovered a MAD-box transcription factor from *Poncirus trifoliata* that was considered to be *PtFLC*. The expression pattern of *PtFLC* was correlated with flowering regulation of precocious trifoliate orange and associated with the transition from juvenile to mature trees. Later, *PtELF5*, a floral repressor with an expression pattern correlated with the seasonal periodicity of flowering, was identified (Zhang and others 2011). In *Arabidopsis*, *AtFLC* is regulated by *AtELF5*, which also acts as a floral repressor. However, the *AtELF5* expression pattern was opposite that of *AtFLC* and *PtFLC* in transgenic and wild-type plants at the flowering stage, supporting the conclusion that floral induction and flowering in precocious *P. trifoliata* is attributable to the expression of *PtELF5*.

Considering that *SOC1* regulates *LFY* [see review by Lee and Lee (2010)], it is not surprising that GA_3 does not modify *CsLFY* expression in leaves compared with the control. However, the application of PBZ at the inductive season significantly boosted the relative expression of *CsLFY* in leaves, particularly at bud break. *Citrus* species have different types of shoots: some are single-flowered while others develop numerous flowers from an inflorescence meristem. In addition, all buds are multimeristematic buds (Davenport 1990) and, therefore, several shoots per bud can develop (Guardiola and others 1982). Taken together, *Citrus* trees develop single- and multiflowered leafy and leafless shoots and vegetative shoots, and some of them may originate from the same bud (multisprouted buds) (Guardiola and others 1982). Because all meristems of a given bud are under the same environmental inductive conditions, the different types of shoots must be established by endogenous factors such as fruit load, bud location on the tree and on the shoot, and so on. On the other hand, among the known genes involved in the induction of flowering in *Citrus*, *SOC1* likely promotes the switch from a vegetative meristem to an inflorescence meristem, while *CsLFY* and *CsAP1* induce the formation of the floral meristem (Liu and others 2009; Pillitteri and others 2004a). However, differences in the number of shoots developed per 100 nodes due to the application of GA_3 apparently do not match up with the relative expression of *SOC1*. This is in accordance with the differences observed in the number of sprouted buds rather than in the type of shoot as a result of GA_3, that is, GA_3 does not affect the production of different types of shoot (all kind of shoots, single- and multiflowered leafy and leafless shoots, were present on treated trees; see Table 2) but affects the number of shoots. At present, it is unknown if mature tree buds that do not sprout due to GA_3 treatment can express flowering genes, although it appears that the GA_3 effect of reducing bud sprouting selectively affects those buds producing inflorescences (Guardiola and others 1982; Table 2). Furthermore, there were no significant differences in the number of flowers per shoot, in both leafy and leafless inflorescences, due to GA_3, which is in agreement with the fact that GA_3 has no effect on the relative expression of *CsLFY* in leaves, whereas the PBZ positive effect on increasing the number of flowers per inflorescence, in both leafy and leafless inflorescences, paralleled an increase in the relative expression of *CsLFY* in leaves. The effect of GA_3 inhibiting bud sprouting and a positive relationship between the number of new shoots and flowering have been reported for *Citrus* (García-Luis and others 1986) and other woody perennials (Reig and others 2011).

Our results show that *CsLFY* transcripts in control leaves increased, coinciding with floral bud differentiation. This is in agreement with previous results on Satsuma mandarin (Nishikawa and others 2007) and on the 'Moncada' mandarin hybrid (Muñoz-Fambuena and others

2011). Later, there was a reduction in its relative expression coinciding with an increase in the relative expression of *CsAP1*. This agrees with Nishikawa and others (2007) who reported a decrease of *CsLFY* transcripts for Satsuma mandarin leaves coinciding with an increase in *CsAP1* transcripts at the end of the floral bud inductive period. In addition, it has been reported that *LFY* directly regulates the expression of *AP1* (William and others 2004). For both genes, GA_3 did not significantly modify their relative expression in leaves. This seems logical because *CsAP1* is involved in floral organ development, as suggested by Pillitteri and others (2004a), and floral organs develop in all trees regardless of treatment, but differ in number. This may also be because the level of *CsLFY* expression is not reaching the critical threshold (Blázquez and others 1997), which, in turn, might explain why PBZ did not affect *CsAP1* significantly.

In conclusion, our results with sweet orange strongly suggest that GA_3 inhibits flowering by repressing *CiFT* expression in leaves when applied during the floral bud inductive period, contributing to our knowledge of the molecular mechanism underlying the GA effect in controlling flowering of woody fruit tree species.

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References

Ayalon S, Monselise SP (1960) Flower bud induction and differentiation in the Shamouti orange. Proc Am Soc Hortic Sci 75:216–221

Bertelsen MG, Tustin DS, Waagepetersen RP (2002) Effects of GA_3 and GA_{4+7} on early bud development of apple. J Hortic Sci Biotechnol 77:83–90

Blázquez MA, Soowal L, Lee I, Weigel D (1997) LEAFY expression and flower initiation in *Arabidopsis*. Development 124:3835–3844

Boss PK, Thomas MR (2002) Association and dwarfism and floral induction with a grape 'green revolution' mutation. Nature 416:847–850

Boss PK, Buckeridge EJ, Poole A, Thomas MR (2003) New insights into grapevine flowering. Funct Plant Biol 30:593–606

Boss PK, Sreekantan L, Thomas MR (2006) A grapevine TFL1 homologue can delay flowering and alter floral development when over expressed in heterologous species. Funct Plant Biol 33:31–41

Bustin SA (2002) Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29:23–39

Davenport TL (1990) Citrus flowering. Hortic Rev 12:349–408

Davenport TL (2000) Leaves are not necessary for citrus floral induction. Proc Int Soc Citric 1:660–661

Endo T, Shimada T, Fujii H, Kobayashi Y, Araki T, Omura M (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

Esumi T, Tao R, Yonemori K (2005) Isolation of LEAFY and TERMINAL FLOWER 1 homologues from six fruit tree species in the subfamily Maloideae of the Rosaceae. Sex Plant Reprod 17:277–287

Fatta del Bosco G (1961) Indagini sull'epoca di differenziazione delle gemme nel nespolo del Giappone. Riv Ortoflorofrut Ital 2:104–118

García-Luis A, Almela V, Monerri C, Agustí M, Guardiola JL (1986) Inhibition of flowering in vivo by existing fruits and applied growth regulators in *Citrus unshiu*. Physiol Plant 66:515–520

García-Pallás I, Blanco A (2001) The inhibition of flower bud differentiation in 'Crimson Gold' nectarine with GA_3 as an alternative to hand thinning. Sci Hortic 90:265–278

González-Rossia D, Juan M, Reig C, Agustí M (2006) The inhibition of flowering by means of gibberellic acid application reduces the cost of hand thinning in Japanese plums (*Prunus salicina* Lindl.). Sci Hortic 110:319–323

Guardiola JL, Agustí M, García-Marí F (1977) Gibberellic acid and flower bud development in sweet orange. Proc Int Soc Citric 2:696–699

Guardiola JL, Monerri C, Agustí M (1982) The inhibitory effect of gibberellic acid on flowering in Citrus. Physiol Plant 55:136–142

Hartmann HT, Fadl MS, Hackett WP (1967) Initiation of flowering and changes in endogenous inhibitors and promoters in olive buds as a result of chilling. Physiol Plant 20:746–759

Harty AR, van Staden J (1988) Paclobutrazol and temperature effects on lemon. Proc Int Soc Citric 1:343–353

Hashimoto JG, Beadles-Bohling AS, Wiren KM (2004) Comparison of RiboGreen and 18S rRNA quantitation for normalizing real-time RT-PCR expression analysis. BioTechniques 36:54–60

Koshita Y, Takahara T, Ogata T, Goto A (1999) Involvement of endogenous plant hormones (IAA, ABA, GA) in leaves and flower bud formation of Satsuma mandarin (*Citrus unshiu* Marc.). Sci Hortic 79:185–194

Kotoda N, Wada M, Komori S, Kidou S, Abe K, Masuda T, Soejima J (2000) Expression pattern of homologues of floral meristem identity genes LFY and AP1 during flower development in apple. J Am Soc Hortic Sci 125:398–403

Lee J, Lee I (2010) Regulation and function of SOC1, a flowering pathway integrator. J Exp Bot 61:2247–2254

Lenahan OM, Whiting MD, Elfving DC (2006) Gibberellic acid inhibits floral bud induction and improves 'Bing' sweet cherry fruit quality. Hort Sci 41:654–659

Liu C, Xi WY, Shen LS, Tan CP, Yu H (2009) Regulation of floral patterning by flowering time genes. Dev Cell 16:711–722

Luckwill LC (1970) The control of growth and fruitfulness of apple trees. In: Luckwill LV, Cutting CV (eds) Physiology of tree crops. Academic Press, New York, pp 237–254

Martínez-Fuentes A, Mesejo C, Juan M, Almela V, Agustí M (2004) Restrictions on the exogenous control of flowering in Citrus. Acta Hortic 632:91–98

Martínez-Fuentes A, Mesejo C, Reig C, Agustí M (2010) Timing of the inhibitory effect of fruit on return bloom of 'Valencia' sweet orange (*Citrus sinensis* (L.) Osbeck). J Sci Food Agric 90:1936–1943

Monselise SP, Halevy AH (1964) Chemical inhibition and promotion of citrus flower bud induction. Proc Am Soc Hortic Sci 84: 141–146

Monselise SP, Goren R, Halevy AH (1966) Effect of B-nine, cycocel and benzothiazole oxyacetate on flower bud induction of lemon trees. Proc Am Soc Hortic Sci 89:195–200

Moss GI (1971) Effect of fruit on flowering in relation to biennial bearing in sweet orange (*Citrus sinensis*). J Hortic Sci 46: 177–184

Muñoz-Fambuena N, Mesejo C, González-Mas MC, Primo-Millo E, Agustí M, Iglesias D (2011) Fruit regulates seasonal expression

of flowering genes in alternate bearing 'Moncada' mandarin. Ann Bot 108:511–519

Mutasa-Göttgens E, Hedden P (2009) Gibberellin as a factor in floral regulatory networks. J Exp Bot 60:1979–1989

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

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

Peña L, Martín-Trillo M, Juárez J, Pina JA, Navarro L, Martínez-Zapater JM (2001) Constitutive expression of *Arabidopsis LEAFY* or *APETALA1* genes in citrus reduces their generation time. Nat Biotechnol 19:263–267

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

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

Reig C, Farina V, Volpe G, Mesejo C, Martínez-Fuentes A, Barone F, Calabrese F, Agustí M (2011) Gibberellic acid and flower bud development in loquat (*Eriobotrya japonica* Lindl.). Sci Hortic 129:27–31

Salazar-García S, Lovatt CJ (1998) GA_3 application alters flowering phenology of 'Hass' avocado. J Am Soc Hortic Sci 123:791–797

Searle I, He Y, Turck F, Vincent C, Fornara F, Brober S, Amasino RA, Coupland G (2006) The transcription factor FLC confers a flowering response to vernalization by repressing meristem competence and systemic signaling in *Arabidopsis*. Genes Dev 20:898–912

Singh LB (1959) Movement of flowering substances in the mango (*Mangifera indica* L) leaves. Hortic Adv 3:20–27

Sung SK, Yu GH, An G (1999) Characterization of MdMADS2, a member of the SQUAMOSA subfamily of genes in apple. Plant Physiol 120:969–978

Sung SK, Yu GH, Nam J, Jeong DH, An G (2000) Developmentally regulated expression of two MADS-box genes, MdMADS3 and MdMADS4, in morphogenesis of flower buds and fruit in apple. Planta 210:519–528

Terol J, Naranjo Ma , Ollitrault P, Talon M (2008) Development of genomic resources for *Citrus clementina*: Characterization of three deep-coverage BAC libraries and analysis of 46,000 BAC end sequences. BMC Genomics 9:423

Wilkie JD, Sedgley M, Olesen T (2008) Regulation of floral initiation in horticultural trees. J Exp Bot 59:3215–3228

William DA, Su Y, Smith MR, Lu M, Baldwin DA, Wagner D (2004) Genomic identification of direct target genes of LEAFY. Proc Natl Acad Sci U S A 101:1775–1780

Wilson RN, Heckman JW, Somerville CR (1992) Gibberellin is required for flowering in *Arabidopsis thaliana* under short days. Plant Physiol 100:403–408

Yan J, Yuan F, Long G, Qin L, Deng Z (2012) Selection of reference genes for quantitative real-time RT-PCR analysis in citrus. Mol Biol Rep 39(2):1831–1838

Ying Z, Davenport TL (2004) Leaves required for floral induction of lychee. Plant Growth Regul Soc Am Q 32:132–137

Zhang L, Xu Y, Ma R (2008) Molecular cloning, identification, and chromosomal localization of two MADS box genes in peach (*Prunus persica*). J Genet Genomics 35:365–372

Zhang JZ, Li ZM, Mei L, Yao JL, Hu CG (2009) *PtFLC* homolog from trifoliate orange (*Poncirus trifoliata*) is regulated by alternative splicing and experiences seasonal fluctuation in expression level. Planta 229:847–859

Zhang JZ, Ai XY, Sun LM, Zhang DL, Guo WW, Deng XX, Hu CG (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