Fruit load modulates flowering-related gene expression in buds of alternate-bearing 'Moncada' mandarin

Natalia Muñoz-Fambuena[1], Carlos Mesejo[1], M. Carmen González-Mas[2], Eduardo Primo-Millo[2], Manuel Agustí[1] and Domingo J. Iglesias[2],*

[1]*Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, E-46022 Valencia, Spain and* [2]*Centro de Citricultura y Producción Vegetal, Instituto Valenciano de Investigaciones Agrarias, E-46113 Moncada, Valencia, Spain*
For correspondence. E-mail iglesias_dom@gva.es

• *Background and Aims* Gene determination of flowering is the result of complex interactions involving both promoters and inhibitors. In this study, the expression of flowering-related genes at the meristem level in alternate-bearing citrus trees is analysed, together with the interplay between buds and leaves in the determination of flowering.
• *Methods* First defruiting experiments were performed to manipulate blossoming intensity in 'Moncada' mandarin, *Citrus clementina*. Further defoliation was performed to elucidate the role leaves play in the flowering process. In both cases, the activity of flowering-related genes was investigated at the flower induction (November) and differentiation (February) stages.
• *Key Results* Study of the expression pattern of flowering-genes in buds from *on* (fully loaded) and *off* (without fruits) trees revealed that homologues of *FLOWERING LOCUS T* (*CiFT*), *TWIN SISTER OF FT* (*TSF*), *APETALA1* (*CsAP1*) and *LEAFY* (*CsLFY*) were negatively affected by fruit load. *CiFT* and *TSF* activities showed a marked increase in buds from *off* trees through the study period (ten-fold in November). By contrast, expression of the homologues of the flowering inhibitors of *TERMINAL FLOWER 1* (*CsTFL*), *TERMINAL FLOWER 2* (*TFL2*) and *FLOWERING LOCUS C* (*FLC*) was generally lower in *off* trees. Regarding floral identity genes, the increase in *CsAP1* expression in *off* trees was much greater in buds than in leaves, and significant variations in *CsLFY* expression (approx. 20 %) were found only in February. Defoliation experiments further revealed that the absence of leaves completely abolished blossoming and severely affected the expression of most of the flowering-related genes, particularly decreasing the activity of floral promoters and of *CsAP1* at the induction stage.
• *Conclusions* These results suggest that the presence of fruit affects flowering by greatly altering gene-expression not only at the leaf but also at the meristem level. Although leaves are required for flowering to occur, their absence strongly affects the activity of floral promoters and identity genes.

Key words: Alternate bearing, *AP1*, citrus, flowering, *FT*, *FD*, *FLC*, *LFY*, *SOC1*, *TFL1*, *TFL2*, *TSF*.

INTRODUCTION

Under Mediterranean climatic conditions, *Citrus* floral evocation takes place in late autumn whereas differentiation and morphogenesis occur later, around early spring (Sherman and Beckman, 2003). *Citrus* species have a tropical–subtropical evergreen nature and, unlike deciduous fruit trees, their buds do not exhibit true dormancy, remaining in rest until initiation. The resting bud itself consists of an undifferentiated apical meristem, and initiation begins with cell division and differentiation of the primordium surrounding the meristematic dome (see revisions by Schneider, 1968; Lord and Eckard, 1985; Davenport, 1990). Previous research has demonstrated that fruit load acts as a strong inhibitor of flowering in many fruit-tree species, including citrus. This inhibition is determined by several factors such as environmental conditions, cultivar, number of fruits per tree and harvesting date (Martínez-Fuentes *et al.*, 2010). Nutritional and hormonal factors also seem to be involved in the metabolic and biochemical pathways leading to fruit load-mediated inhibition of

flowering (Koshita *et al.*, 1999). Thus, seasonal changes in carbohydrate composition of buds or the mineral status of the tree are physiologically connected to flower differentiation (Vemmos, 1999; Yahata *et al.*, 2004). At the molecular level, gene determination of flowering appears to be the result of complex interactions that involve both promoter and inhibitor genes in the context of an integrative system. Thus, specific homologues of *FLOWERING LOCUS T* (*FT*) (*CiFT*, Endo *et al.*, 2005), *TERMINAL FLOWER 1* (*CsTFL*, Pillitteri *et al.*, 2004*a*), *LEAFY* (*LFY*) and *APETALA* (*AP1*) (*CsLFY* and *CsAP1*, Pillitteri *et al.*, 2004*b*), and *SUPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*) (*CsSL1*, Tan and Swain, 2007) have been previously isolated and characterized in *Citrus*. Moreover, a genetic framework for flowering-time pathways in *Citrus* has been proposed, and overviews of the relationships amongst the main genes involved have been given (Dornelas *et al.*, 2007*a*, *b*). Additional effort has been recently made by our group to elucidate the molecular basis of floral regulation by fruit load (Muñoz-Fambuena *et al.*, 2011), and we proposed that the promoter gene *CiFT*

plays a pivotal role in the inhibition of flowering as a consequence of crop load through repressive mechanisms. Other promoter genes, such as *SOC1*, appear to exhibit minor responses to the presence of fruit. Hormonal compounds such as gibberellins have also been demonstrated to participate in the regulation processes (Muñoz-Fambuena *et al.*, 2012). On the other hand, expression in leaves of those homologues of *AP1* and *LFY* is strongly modulated by fruit load, although their activity appears to be more closely associated with floral identity. By contrast, inhibitors such as *TFL1* and *FLOWERING LOCUS C* (*FLC*) do not appear to be correlated with fruit load.

However, although most of the studies have focused on the changes of gene activity at the leaf level, some recent studies indicate that the meristem might play a central role in the molecular determination of flowering. As large numbers of flowering-related gene transcripts have been described at this location, some authors have remarked on the importance of apices in controlling flowering time (Jang *et al.*, 2009). Thus, some studies indicate that homologues of *FT* and *SOC1* are expressed in the primordia of various species, and some others show that many organs of the plant are active producers of the associated proteins (Sreekantan and Thomas, 2006 in grapevine; Esumi *et al.*, 2009 in apricot; Kotoda *et al.*, 2010 in apple). Regarding identity genes, because the bud is the physical location for floral differentiation, more conventional research highlights the role and importance of gene expression at this level. Thus, it has been reported that floral identity genes are mainly expressed in the floral meristems of numerous plant species (Southerton *et al.*, 1998 in *Eucalyptus*; Rottmann *et al.*, 2000 in *Populus*; Wada *et al.*, 2002 in apple; Yu *et al.*, 2005 in papaya). In citrus, preliminary investigations have demonstrated that leaves are not exclusive in the production of transcripts of flowering-related genes (Nishikawa *et al.*, 2007). However, there is a lack of information regarding the intensity, role and relative importance of the expression of flowering-related genes at the meristem level, and to what extent buds and leaves cooperate in flowering determination as a consequence of fruit bearing.

In addition, because *CiFT* appears to be the central promoter regulating flowering in alternate-bearing citrus, and *CsAP1* and *CsLFY* represent the main floral identity genes determining bud fate, we extend our study to other floral integrator genes, which modulate and control their function at the apex level in model species. Thus, paralogues of *FT* have been reported to contribute greatly to the production of the small globular FT protein, associated with the mythic florigen. This is the case of *TWIN SISTER OF FT* (*TSF*), the closest homologue of *FT* in *Arabidopsis*, which probably constitutes another independent and direct regulatory target of *CONSTANS* (Yamaguchi *et al.*, 2005). Although *TSF* is reported to have similar functions to *FT*, it acts independently in flowering determination (Yu *et al.*, 2006) and therefore it may play an important role in FT protein supply to meristems in both leaves and buds. Similarly, *FLOWERING LOCUS D* (*FD*) has been described to encode a bZIP protein required for *FT* function, and has recently been described to take part in the specific signalling pathways that occur at the shoot apex (Abe *et al.*, 2005). Finally, *TERMINAL FLOWER 2* (*TFL2*), which was defined over a decade ago as a main

controller of the reproductive transition and meristem identity in *Arabidopsis*, is known to participate in flower induction and bud differentiation (Larsson *et al.*, 1998). It encodes an HP1-like protein that negatively regulates *FT* expression and affects floral identity activity (Takada *et al.*, 2002). Although the expression dynamics of those genes would appear to be very relevant to developing *FT* function and modulating floral morphogenetic processes, it is completely unknown in most tree species. Here we endeavour to shed light on the regulation of *FT* activity and floral identity at the meristem level by studying those complementary but necessary genes, and thus provide further insight into the underlying mechanisms that fine-tune *FT* function and floral identity in alternate-bearing citrus.

In summary, previous research has been conducted into the role of the main flowering promoters and inhibitors in citrus trees, but there is a complete lack of knowledge about the role and importance of buds in the regulation process. We investigate the role this tissue plays in flowering-related gene expression through the manipulation of flowering intensity and/or the presence of leaves. In addition, because a pivotal role has been proposed for some genes that strongly modulate the action of *FT* and cooperate with floral identity genes at the bud level in model species, we extend our study to the changes in expression of the putative orthologues in citrus.

MATERIAL AND METHODS

Plant material

This study was conducted with 12-year-old field-grown trees of 'Moncada' mandarin {Clementina Oroval (*Citrus clementina* Hort. ex Tan.) × 'Kara' mandarin [*C. unshiu* (Swingle) Marcow. × *C. nobilis* Lour.]} trees, grafted onto Carrizo citrange [*C. sinensis* Osbeck × *Poncirus trifoliata* (L.) Raf.] rootstock, planted 5 m × 5 m apart in a loamy clay soil, with drip irrigation. Experimental fields were located in the IVIA Research Station (Moncada, Spain). Trees of this cultivar exhibit a marked alternate-bearing behaviour.

Defruiting experiments were performed on a set of 12 trees, and two levels of fruit removal were employed (0 and 100 %, six trees per treatment). Defoliation treatments were performed on another 12 either fully loaded or unloaded trees (0 and 100 % leaf removal, six trees per treatment). Leaves were carefully removed to avoid bud damage and all of them started to develop the following spring. Both experiments were performed at the onset of the stage II of fruit development (July). Trees were selected for homogeneity in diameter, canopy height, size and shape and a randomized complete-block design was employed. In mid November and at the end of February, fully developed mature adult leaves (*n* = 30 per tree) and non-differentiated buds (≥100 mg f. wt per tree; see Fig. 1) from *on* (fully loaded) and *off* trees (without fruits) were collected for RNA extraction. Samples were immediately ground and stored at −80 °C until analysis.

Flowering evaluation

Flowering intensity was evaluated in spring considering four branches per tree of three ages (late spring, summer and

autumn sprouts) with some 300 nodes per branch. Numbers of both sprouted nodes and sprouts were counted. The flowers per sprout were also counted, with the results given as the number of flowers per 100 nodes to compensate for the differences in size of the selected branches. Additionally, total yield per tree was determined by counting and weighing all fruits at harvest (April).

RNA extraction and RT-PCR

Total RNA was isolated from frozen tissue using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA samples were treated with RNase-free DNase (Qiagen) through column purification following the manufacturer's

FIG. 1. Detail of the buds sampled in mid November and at the end of February for gene-expression analyses. *Citrus* buds, unlike those of deciduous fruit trees, do not exhibit a true dormancy, remaining in rest until initiation. The resting bud itself contains an undifferentiated primordium surrounding the meristematic dome.

instructions. RNA quality was tested based on OD_{260}/OD_{280} and gel electrophoresis. RNA concentration was determined by fluorometric assays with the RiboGreen dye (Molecular Probes, Engene, 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 version 4·0. One-step RT-PCR was carried out. Reactions contained 2·5 U MultiScribe reverse transcriptase (Applied Biosystems, Carlsbad, CA, USA), 1 U RNase Inhibitor (Applied Biosystems), 2 µL 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 µL. Incubations were carried out at 48 °C for 30 min and 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. Fluorescence intensity data were acquired during the 72 °C extension step and transformed to relative mRNA values using a ten-fold dilution series of RNA samples as the standard curve. Relative mRNA levels were then normalized to total mRNA amounts (Bustin, 2002; Hashimoto *et al.*, 2004) and, in each case, an expression value of 1 was arbitrarily assigned to the sample collected in November corresponding to the buds from intact *on* trees. Beta actin was used as the reference gene, according to Yan *et al.* (2012). Specificity of the amplification reactions was assessed using post-amplification dissociation curves and by sequencing the reaction product.

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

TABLE 1. *List of primers used for quantitative real-time PCR*

Annotation	EST code*	5′-Direct primer-3′ 5′-Reverse primer-3′	Predicted product (bp)
CiFT	aCL6275Contig1	GGGAGGCAGACTGTTTATGC CGGAGGTCCCAGATTGTAAA	84
CsTFL	aCL6873Contig1	TCCGTCCACAGTTGTTTCAA TCACTAGGGCCAGGAACATC	105
CsAP1	aCL2870Contig1	CAAAACCAGGTTCCCAACAC ACGAACATACGGGTTCAAGG	139
CsLFY	aC34107C06EF_c	TCTTGATCCAGGTCCAGAACATC TAGTCACCTTGGTTGGGCATT	63
CsSL1	aC31703B03EF_c	TCAGCTTCTTCCCATTTTGG AAGGTGACTTGCCTGCTTGT	135
FLC	aC01009F11SK_c	TCTTTGGTGCAGACACAACTG TCTTCACTCAGCAGCTTTTCC	74
TSF	aIC0AAA15CC06RM1_c	TGCATGCAGGCCAAGAGATTGTGA TGCCTTCCAAGTTGCCGGAACAA	97
FD	aCL3553Contig1	TGGAAGAAGTTTGGCAGGA TGTTTGGGATGGCAGTGTT	69
TFL2	aKN0AAP8YL12FM1_c	GCGGCCGAAGCTTGATGAAGGATT TGCGAACCCTTTTCCGACGAATGG	62

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

Statistical analyses

The experiment was laid out in randomized blocks, with single-tree plots and six replicates per treatment. Parameters were statistically tested by analyses of variance (ANOVA), using the least significant differences (LSD) test for separation of means. When required, percentages were arcsin-transformed to homogenize the variance. StatGraphics Plus software for Windows, version 5·1 (Statistical Graphics, Englewood Cliffs, NJ, USA), was used.

RESULTS

Effect of fruit load on flowering

The effect of fruit load on flowering is shown in Fig. 2. The removal of all fruits severely affected fruit load. Thus, the presence of fruit drastically inhibited the number of flowers produced in spring (1·3 vs. 153·6 flowers/100 nodes in *on* and *off* trees, respectively). The effects were also evident when crop load was examined the following year: those *off* trees produced a mean of 104·1 kg fruit per tree, in contrast to the nil yield produced by *on* trees.

Expression of flowering-related genes

CiFT and *TSF* expression was strongly affected by fruit load throughout the study period (Fig. 3). Significant differences in the number of mRNA transcripts were detected between *on* and *off* trees in both buds and leaves. Thus, the activity registered in *off* trees in November was more than ten-fold higher than that in *on* trees in both tissues. In February, although still statistically significant, differences in buds decreased notably (about five-fold), whereas in leaves increased up to 17-fold. *TSF* expression dynamics strongly paralleled that of *CiFT*, and *off* trees presented a significantly higher number of transcripts in all cases. Differences between *on* and *off* trees were greater in buds than in leaves. Thus, differences in buds ranged from 4·9- to 2·5-fold in November and

February respectively, whereas leaves ranged from 1·9- to 1·5-fold on the same dates.

In buds, relative expression of *CsSL1* and *FD* was not affected by fruit load. Interestingly, *CsSL1* expression level in buds and leaves was similar, whereas *FD* activity was much higher at the meristem (about five- and three-fold higher in November and February, respectively).

Expression of the inhibitors, although not always significant, exhibited a systematic decline in buds from *off* trees (Fig. 4).
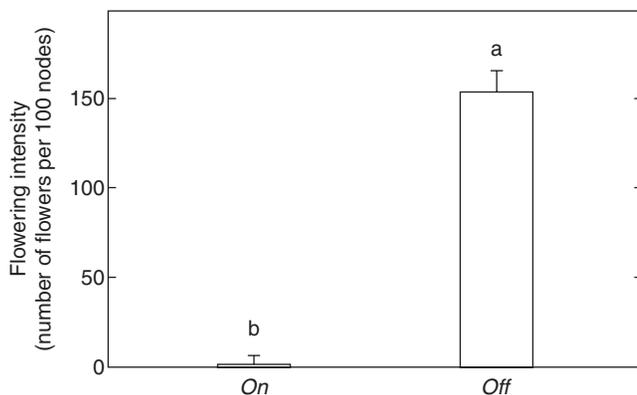

FIG. 2. Effect of fruit removal on flowering intensity of 'Moncada' mandarin trees (*on*: fully loaded; *off*: without fruit). Defruiting treatment consisted of removing the whole yield in the selected trees at the onset of the previous fruit development stage II (July). Data are the means ± s.e. of six trees per treatment and different letters indicate significant differences (*P* ≤ 0·05).
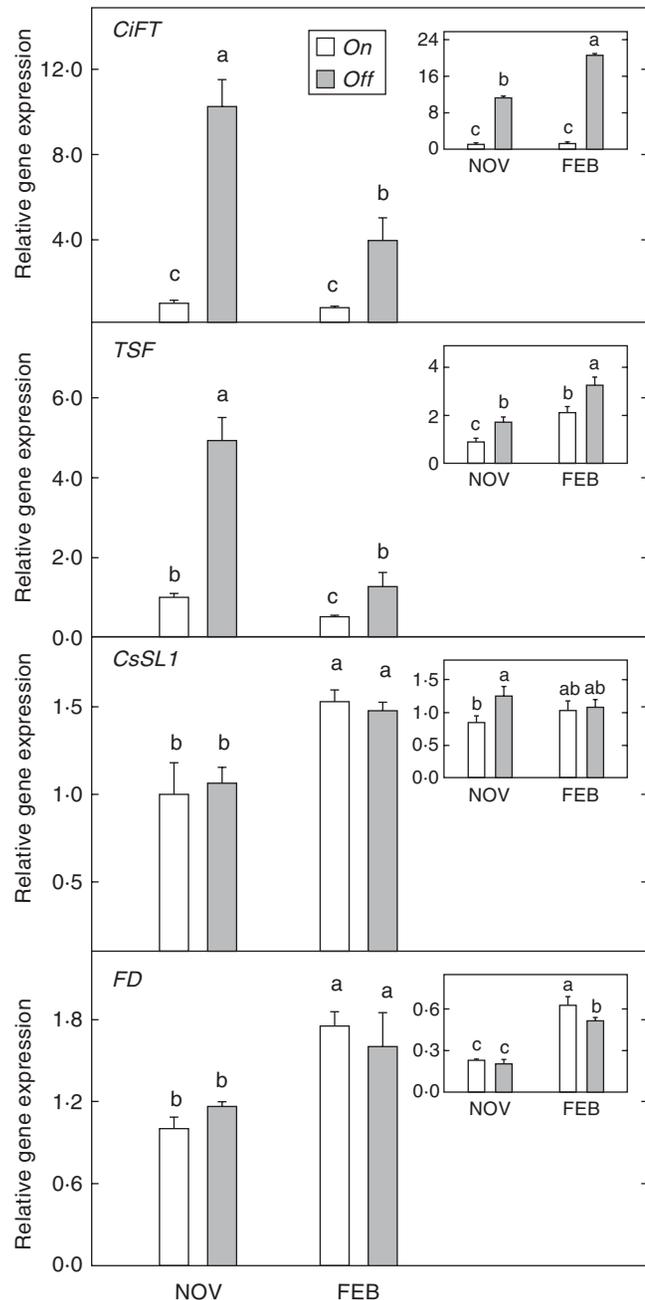

FIG. 3. Expression of flowering promoters (*CiFT*, *TSF*, *CsSL1*, *FD*) in buds from fully loaded (*on*) and without-fruit (*off*) 'Moncada' mandarin trees in November and February. Insets: activity in leaves. Data are means ± s.e. of three independent replicates (*n* = 3). Different letters indicate statistically significant differences (*P* ≤ 0·05).
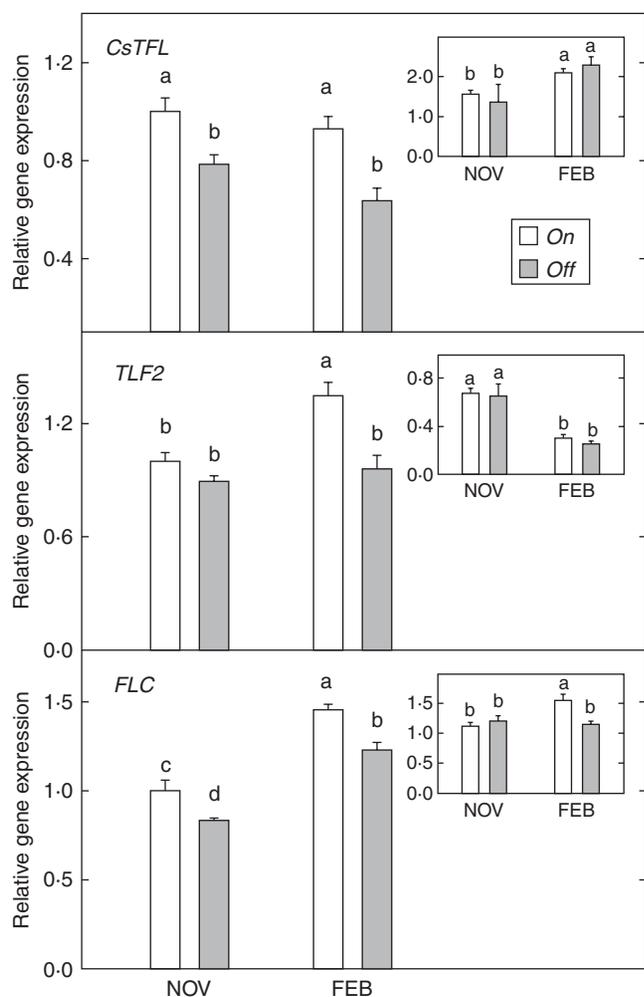
FIG. 4. Expression of flowering inhibitors (*CsTFL1*, *TFL2*, *FLC*) in buds from fully loaded (*on*) and without-fruit (*off*) 'Moncada' mandarin trees in November and February. Insets: activity in leaves. Data are means ± s.e. of three independent replicates (*n* = 3). Different letters indicate statistically significant differences (*P* ≤ 0·05).
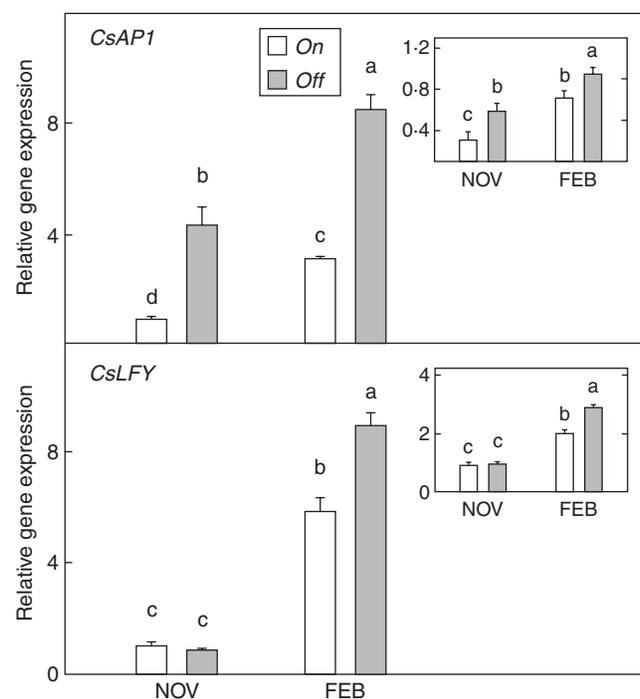

FIG. 5. Expression of floral identity genes (*CsAP1*, *CsLFY*) in buds from fully loaded (*on*) and without-fruit (*off*) 'Moncada' mandarin trees in November and February. Insets: activity in leaves. Data are means ± s.e. of three independent replicates (*n* = 3). Different letters indicate statistically significant differences (*P* ≤ 0·05).

Thus, the expression of *CsTFL* in this tissue was significantly lower in *off* trees in both November and February (22 and 32 %, respectively). A similar pattern was observed for *TFL2* expression in buds, although differences were only significant in February. *CsTFL* and *TFL2* expression were not significantly different in leaves from *on* and *off* trees, irrespective of the sample date. Finally, *FLC* expression in buds from *off* trees exhibited a maintained, significant decline throughout the study period (about 16 % lower).

The activity of floral identity genes in buds and leaves revealed remarkable differences between *on* and *off* trees (Fig. 5). On the one hand, *CsAP1* expression in both tissues greatly increased from November to February, and the accumulation of transcripts was markedly higher in buds than in leaves. Through the study period, those buds from *off* trees showed significantly higher expression values than those from *on* trees, ranging from 2·7- to 4·3-fold higher. Differences between *on* and *off* trees in leaves were minor, although the trend was similar. *CsLFY*, like *CsAP1*, exhibited

higher expression levels in buds than in leaves. Significant changes due to fruit load were detected only in February in both tissues; by this time, activity had increased more than 50 % in buds from *off* trees.

Effect of defoliation on flowering and gene expression

Defoliation had a dramatic effect on flowering in spring. Those trees whose leaves were fully removed dramatically abolished flower production: neither *on* nor *off* trees flowered in spring. In addition, defoliation experiments revealed that the absence of leaves severely affected the expression of several pivotal flowering-related genes (Figs 6 and 7). Thus, bud *CiFT* expression was markedly reduced in defoliated (Fig. 6A) compared with non-defoliated (Fig. 3) *off* trees throughout the study period (15- and three-fold in November and February, respectively). Interestingly, differences in gene activity between *on* and *off* trees only reached significance in November. By this time, expression in buds from defoliated *off* plants was severely reduced with respect to *on* planbts (close to 60 %). An analogous tendency was observed when *TSF* expression was examined. Activity in buds from defoliated trees was significantly reduced (Fig. 6A) with respect to control intact trees (Fig. 3) and, like *FT*, only in November did defoliated *off* trees show a significant decrease in *TSF* expression compared with defoliated *on* trees (about 50 %). Regarding *CsSL1* and *FD*, activity in buds did not show important changes as a consequence of defoliation in November (Figs 3 and 6A). By contrast, *CsSL1* and *FD* expression in
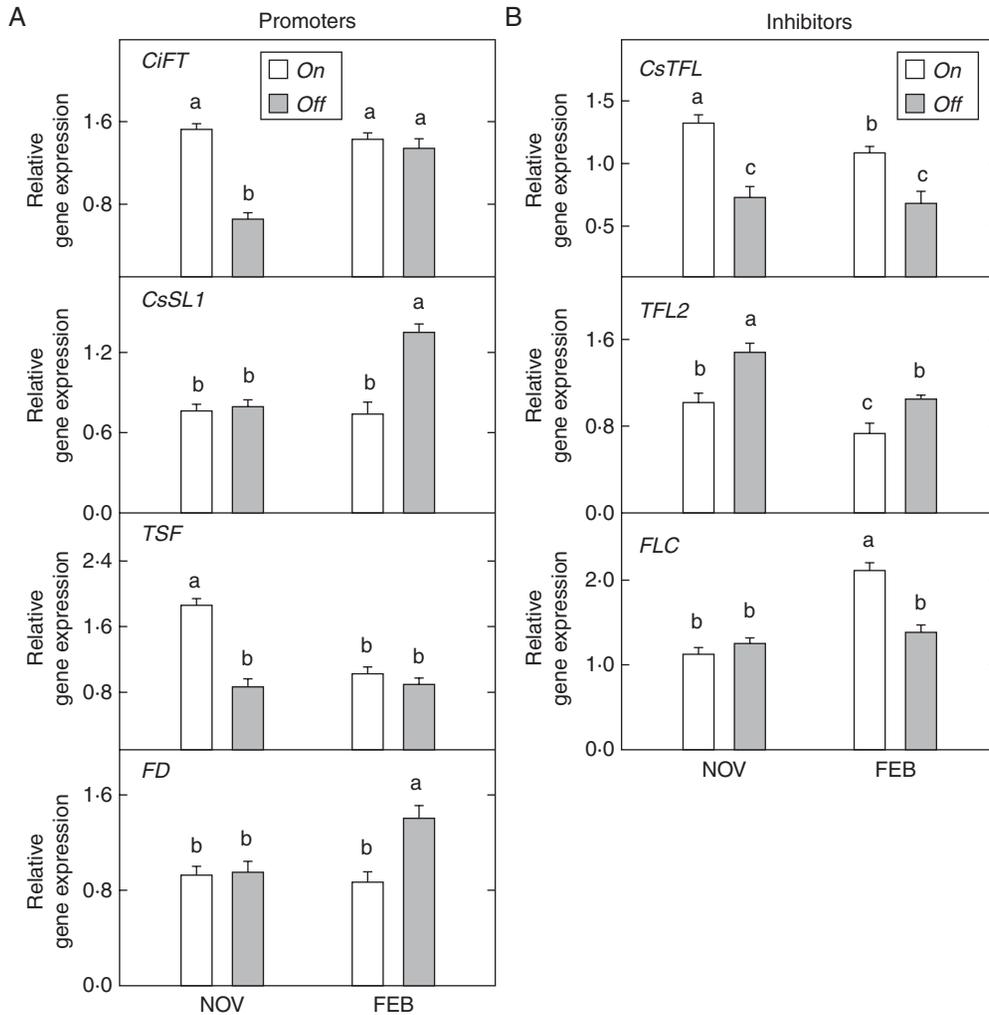
FIG. 6. Effect of defoliation on the expression of floral promoters (A: *CiFT, TSF, CsSL1, FD*) and inhibitors (B: *CsTFL1, TFL2, FLC*) in buds from fully loaded (*on*) and without-fruit (*off*) 'Moncada' mandarin trees in November and February. Data are means ± s.e. of three independent replicates (*n* = 3). Different letters indicate statistically significant differences (*P* ≤ 0·05).

buds of defoliated trees changed as a consequence of fruit load in February in both cases, and at this time *on* trees exhibited low activities compared with *off* trees (about 50 %).

In general, expression intensity of flowering-inhibitors was not noticeably affected by leaf removal (Figs 4 and 6B); however, *on* and *off* trees responded differently in terms of gene expression. Thus, whereas *CsTFL* expression in defoliated *off* trees exhibited a marked decrease with respect to *on* trees in both November and February (45 and 37 %, respectively), *TFL2* followed a significant, opposite trend. *FLC* variations were only observed in February (like control non-defoliated trees, Fig. 4), and by this time expression in *off* plants was significantly reduced (close to 35 %).

The activity of floral identity genes was also noticeably altered as a consequence of leaf removal (Fig. 7). On the one hand, *CsAP1* expression patently decreased in defoliated *off* trees in both November and February compared with control plants (four-fold, see Fig. 5). Differences between *on* and *off* trees were only found in February, buds from defoliated *off* trees showing higher expression values (1·5-fold).

Surprisingly, *CsLFY* expression in buds from defoliated trees showed a marked increase also in November with respect to control foliated trees (more than five-fold, see also Fig. 5). Interestingly, *off* trees exhibited significant increases in *CsLFY* expression values with respect to *on* trees in both November and February, with differences ranging from 28 to 39 %, respectively.

DISCUSSION

Effect of fruit load on flowering

The presence of fruit is commonly described to affect flowering in citrus species (Moss, 1971). In the present study, the number of flowers produced in spring was modulated by manipulating the number of fruits. Thus, non-defruited fully loaded trees did not flower at all, whereas completely defruited trees displayed the highest blossoming intensity, associated with higher fruit load (Fig. 2). The results obtained herein
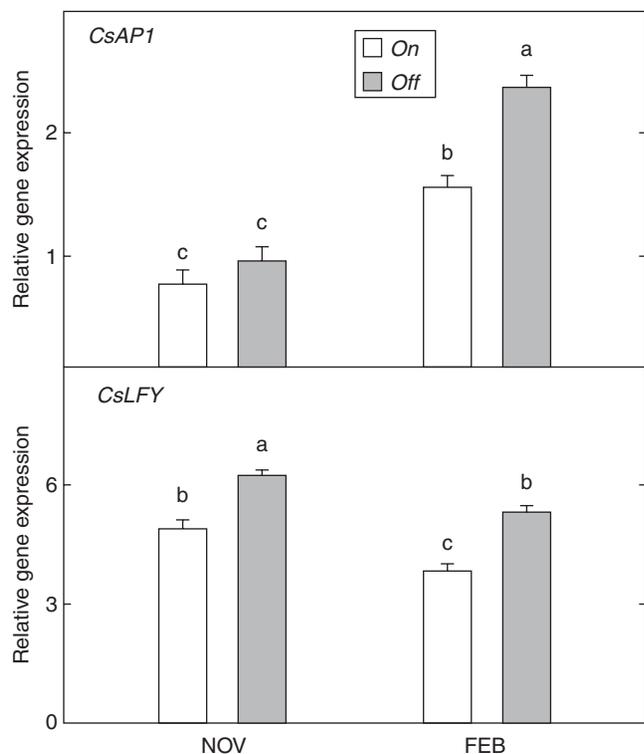
FIG. 7. Effect of defoliation on the expression of floral identity genes (*CsAP1, CsLFY*) in buds from fully loaded (*on*) and without-fruit (*off*) 'Moncada' mandarin trees in November and February. Data are means ± s.e. of three independent replicates ($n = 3$). Different letters indicate statistically significant differences ($P \leq 0.05$).

support previous observations, confirming the effect of alternate bearing, and therefore fruit load, on flowering dynamics.

Previous research has shown that differences in gene expression in leaves arise as a consequence of alternate bearing in citrus trees (Muñoz-Fambuena *et al.*, 2011). The present study shows that active expression of the main genes involved in floral induction and differentiation also occurs in citrus tree buds. Our findings provide further evidence for the control of flowering not only in time but also in space, and shed light on the role of the meristem in the regulation process. Additionally, we describe changes in the activity of several genes that influence the principal flowering pathways at the apical level.

Regulation of the expression of flowering-related genes at the meristem

Regarding the primary response to floral induction, which is highly conserved in plant species, the activation of *FT* has been shown to play a central role in the flowering process (Matsuda *et al.*, 2009; Trankner *et al.*, 2010). In citrus, the corresponding orthologue *CiFT* is a key gene determining flowering (Nishikawa *et al.*, 2010), and previous studies demonstrate that, at least at the leaf level, it exerts rigorous control under alternate-bearing conditions (Muñoz-Fambuena *et al.*, 2011). The results presented herein further indicate that, like leaves, buds from unloaded trees exhibit high *CiFT* activity. This was observed throughout the study period, suggesting that

active, constitutive FT protein production also occurs at the apical level, which finally leads to high flowering intensity in spring. Particularly at the inductive stage (November), the differences between *on* and *off* trees were maximum in both leaves and buds (about ten-fold higher in *off* plants). Later, in February, activity declined in buds compared with leaves and, although still significant, differences between *on* and *off* trees decreased. As expected, the expression pattern registered for *TSF*, the closest homologue of *FT* in *Arabidopsis*, strongly paralleled that of *CiFT* in all cases, although the intensity and the magnitude of the differences between treatments was minor. It is assumed that *TSF* in *Arabidopsis* is a main promoter of the flowering processes together with *FT*, but makes a distinct contribution (Mathieu *et al.*, 2007; Yu *et al.*, 2005). As the greatest differences in *FT* and *TSF* activity between *on* and *off* trees were found in November in both buds and leaves, our data also suggest a critical and complementary role at the floral inductive stage, supporting previous observations in model species (Notaguchi *et al.*, 2008; Jang *et al.*, 2009). Acting together on a different spatial scale, their heightened activity would increase the pool of FT protein necessary to induce flowering at the apex, either directly or through import processes, at least during the stages closest to the inductive period. In fact, recent research suggests a long-distance action for FT protein, involving complex and selective trafficking pathways that physically connect leaves to meristems (Corbesier and Coupland, 2006; Goto, 2006; Jang *et al.*, 2009). Once the bud is induced, the increased *CiFT* expression found in both buds and leaves (Fig. 3) would be responsible for a continuous flux of FT protein to the developing meristems up until the floral morphogenetic phase is initiated (Jaeger *et al.*, 2006; Giakountis and Coupland, 2008).

Other promoter genes, such as *SOC1*, have also been reported to contribute to the promotion of flowering (Tan and Swain, 2007), and previous studies have analysed their participation in the regulation of flowering under alternate-bearing conditions (Muñoz-Fambuena *et al.*, 2011). Our data indicate that the citrus homologue *CsSL1* shows a constant and similar activity in both buds and leaves, suggesting a minor role in relation to fruit load at the apical level. Probably after flowering induction takes place, expression of *CsSL1* not only supports the continuous promoting action of *CiFT* at the shoot apex (Fig. 3), but also participates in the regulation of floral morphogenesis through the activation of identity genes (e.g. *CsLFY*, see Fig. 5). By contrast, unlike *CsSL1*, *FD* expression in buds was markedly higher than in leaves in both November and February. As in other species (Abe *et al.*, 2003), this observation reinforces the hypothesis that its role is decisive at the apical level, possibly because the transition from vegetative to floral meristem mainly occurs there. In fact, this gene has been described as a strong modulator of *FT* action specifically in the meristem (Abe *et al.*, 2005; Jaeger *et al.*, 2006), integrating temporal and spatial signals and mediating promoter and floral identity molecular pathways (Wigge *et al.*, 2005; Li *et al.*, 2008). The continuous expression observed over time coincides with studies in other species that report a constitutive activity in the shoot apex even before floral induction (see Wigge *et al.*, 2005).

In general, differences were revealed in the expression of inhibitors in buds between *on* and *off* trees. Thus, whereas no important effects were observed as a consequence of

alternate bearing in leaves, there was a continuous decline in expression levels in all genes considered in buds from *off* trees during the study period. This observation supports the hypothesis that floral repressors ensure the correct reproductive timing by controlling promoters, and the reduced expression found in buds from *off* trees facilitates the action of promoters, thereby contributing to flowering induction. Indeed, the lower expression levels in buds than in leaves may suggest that the observed changes in *CsTFL* expression are not a direct consequence of fruit load (Muñoz-Fambuena *et al.*, 2011) but may also be associated with juvenility processes and/or with the maintenance and development of vegetative growth (Pillitteri *et al.*, 2004a; Esumi *et al.*, 2010). In contrast to *CsTFL*, *TFL2* expression levels were always higher in buds than in leaves, suggesting a pivotal role for this gene at this location. In fact, *TFL2* was first described as a main controller of both reproductive transition and meristem identity in *Arabidopsis* (Larsson *et al.*, 1998), and appears to actively and constitutively repress *FT* expression throughout development (Takada *et al.*, 2002; Takada and Goto, 2003). We also extend this observation to the fact that activity was much higher in buds than in leaves in February and that, at that moment, differences between *on* and *off* trees were also highly accentuated there. Effects upon morphogenetic processes have also been reported for this gene, strongly influencing the role of identity genes such as *AP1* and contributing to the maintenance of inflorescence meristem identity and floral architecture (Larsson *et al.*, 1998). Finally, it is interesting to note that *FLC* did not display differences in activity between leaves and buds, concomitant with a function associated with flowering regulation through the vernalization pathway (Michaels *et al.*, 2005), possibly at any plant level. Regardless, lower expression levels at the buds may also continuously activate and facilitate the action of promoters (Michaels *et al.*, 2005).

Study of the expression of floral identity genes, *CsAP1* and *CsLFY*, has corroborated their pivotal role at the bud level (Fig. 5), as a higher production of transcripts in this tissue was found compared with leaves. Irrespective of fruit load, *CsAP1* and *CsLFY* activities were significantly higher in buds than in leaves in February, whereas in November this trend was only encountered for *CsAP1*. The increased number of *CsAP1* transcripts found in *off* trees, more notable in buds, suggests a main correlation with flowering, as reported for other species (Jaya *et al.*, 2010). Together with the reduction in the suppressive action of inhibitor genes, promoter genes such as *FT* and/or *FD* probably contribute to the development of floral morphogenesis (Yamamoto *et al.*, 2004; Notaguchi *et al.*, 2008). By contrast, the absence of differences in *CsLFY* activity between *on* and *off* trees in November suggests a more delayed response. In fact, although high expression levels of both genes coincided in the shoot apex with the stage of flower differentiation and morphogenesis, previous studies in citrus report *CsAP1* to be more effective than *CsLFY* in terms of flowering induction (Peña *et al.*, 2001).

Defoliation-induced changes in flowering intensity and gene expression

Because data revealed that buds actively express the main genes involved in flowering-related processes, defoliation experiments were conducted to further investigate the role of leaves in flowering determination. The results presented herein indicate that leaf removal severely affected flowering-gene expression in buds, and resulted in the complete inhibition of blossoming. Therefore, it can be concluded that leaves are necessary for floral induction and differentiation, independently of the alternate-bearing condition and variations in gene expression in buds might play a complementary role at least during the induction and differentiation stages. As expected, given their role in the flowering process, *CiFT* and *TSF* activities were strongly affected by leaf removal (Fig. 6A). Thus, defoliation treatments dramatically inhibited *CiFT* expression in buds from *off* trees, which did not flower after leaf removal, but not in those from *on* trees, which did not flower in either foliated or defoliated trees. The decrease in the activity was much more accentuated in November than in February, confirming the key role of leaves at the floral inductive stage. Treatment also reduced the expression of *TSF*. Although a certain amount of protein is directly produced at the shoot apex it is not enough to induce flowering and the FT protein pool produced by the leaf appears to be essential to flower development (Notaguchi *et al.*, 2008). Other promoter genes, such as *CsSL1* and *FD*, did not show marked responses to defoliation (Figs 3 and 6A). Significant differences were only observed in February, probably associated with a residual and delayed effect of the early inhibition of *FT* and *TSF*. The defoliation experiment also confirmed that the roles of *CsTFL* and *FLC* seem not to be directly associated with fruit load, as those genes did not substantially change their expression pattern in either *on* or *off* trees due to treatment (Figs 4 and 6B). Interestingly, *TFL2* expression pattern was inverted in *on* and *off* trees as a consequence of leaf removal (see Figs 4 and 6B), concomitantly with the decrease in *FT* protein levels (Fig. 6A), reinforcing its inhibitory role in the absence of leaves.

Defoliation also confirmed the main role of identity genes at the bud level (Fig. 7). The data presented herein show that their expression in buds was drastically affected as a consequence of leaf removal. Thus, *CsAP1* expression decreased greatly in *off* trees in both November and February (about four-fold) with respect to control foliated trees. Interestingly, the effect of *CsLFY* on floral identity has been associated with dates around floral differentiation (February, see Fig. 5), whereas leaf removal induced a reduction of *CsLFY* in buds from *on* and *off* leaves in both November and February, suggesting that latent expression of this gene at the inductive stage, after leaf removal, could contribute to the observed inhibition of flowering.

Together, these are the first results to suggest that the presence of fruit not only affects flowering by severely alters gene expression at the leaf and meristem level in alternate-bearing citrus trees. Thus, *CiFT* and *TSF* expression were strongly inhibited in buds as a consequence of fruit load, and expression of floral identity genes (*CsAP1* and *CsLFY*) was considerably reduced. The flowering inhibitors *CsTFL*, *TLF2* and *FLC* generally exhibited lower expression in *off* trees, probably supporting the action of promoter genes. Finally, although leaves are essential for flowering, fruit defoliation strongly reduced the activity of floral promoters *CiFT*, *TSF* and *CsAP1* in buds, especially at the inductive stage.

LITERATURE CITED

Abe M, Yamamoto S, Kobayashi Y, Nakabayashi H, Ichinoki H, Araki T. 2003. Flowering-time gene *FD* encodes a bZIP protein which is required for the function of a floral pathway integrator *FT*. *Plant and Cell Physiology* **44**: pS133.

Abe M, Kobayashi Y, Yamamoto S, *et al*. 2005. *FD*, a bZIP protein mediating signals from the floral pathway integrator *FT* at the shoot apex. *Science* **309**: 1052–1056.

Bustin SA. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology* **29**: 23–39.

Corbesier L, Coupland G. 2006. The quest for florigen: a review of recent progress. *Journal of Experimental Botany* **57**: 3395–3403.

Davenport TL. 1990. Citrus flowering. *Horticultural Reviews* **12**: 349–408.

Dornelas MC, Camargo RLB, Figueiredo LHM, Takita MA. 2007*a*. A genetic framework for flowering-time pathways in *Citrus* spp. *Genetics and Molecular Biology* **30**: 769–779.

Dornelas MC, Luciana R, Camargo B, Berger IJ, Takita MA. 2007*b*. Towards the identification of flower-specific genes in *Citrus* spp. *Plant Physiology* **3**: 761–768.

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 Research* **14**: 703–712.

Esumi T, Hagihara C, Kitamura Y, *et al*. 2009. Identification of an *FT* ortholog in Japanese apricot (*Prunus mume* Sieb. *et* Zucc.). *Journal of Horticultural Science & Biotechnology* **84**: 149–154.

Esumi T, Kitamura Y, Hagihara C, Yamane H, Tao R. 2010. Identification of a *TFL1* ortholog in Japanese apricot (*Prunus mume* Sieb. *et* Zucc.). *Scientia Horticulturae* **125**: 608–616.

Giakountis A, Coupland G. 2008. Phloem transport of flowering signals. *Current Opinion in Plant Biology* **11**: 687–694.

Goto K. 2006. Protein trafficking of *FT/TFL1* and flowering signal transmission. *Comparative Biochemistry and Physiology: A. Molecular & Integrative Physiology* **143**: S167–S167.

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.

Jaeger KE, Graf A, Wigge PA. 2006. The control of flowering in time and space. *Journal of Experimental Botany* **57**: 3415–3418.

Jang S, Torti S, Coupland G. 2009. Genetic and spatial interactions between *FT*, *TSF* and *SVP* during the early stages of floral induction in *Arabidopsis*. *Plant Journal* **60**: 614–625.

Jaya E, Clemens J, Song JC, Zhang HB, Jameson PE. 2010. Quantitative expression analysis of meristem identity genes in *Eucalyptus occidentalis*: AP1 is an expression marker for flowering. *Tree Physiology* **30**: 304–312.

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

Kotoda N, Hayashi H, Suzuki M, *et al*. 2010. Molecular characterization of *FLOWERING LOCUS T*-like genes of apple (*Malus domestica* Borkh.). *Plant and Cell Physiology* **51**: 561–575.

Larsson AS, Landberg K, Meeks-Wagner DR. 1998. The *TERMINAL FLOWER2* (*TFL2*) gene controls the reproductive transition and meristem identity in *Arabidopsis thaliana*. *Genetics* **149**: 597–605.

Li D, Liu C, Shen L, *et al*. 2008. A repressor complex governs the integration of flowering signals in *Arabidopsis*. *Developmental Cell* **15**: 110–120.

Lord EM, Eckard KJ. 1985. Shoot development in *Citrus sinensis* L. (Washington navel orange). I. Floral and inflorescence ontogeny. *Botanical Gazette* **146**: 320–326.

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

Mathieu J, Warthmann N, Kuttner F, Schmid M. 2007. Export of FT protein from phloem companion cells is sufficient for floral induction in *Arabidopsis*. *Current Biology* **17**: 1055–1060.

Matsuda N, Ikeda K, Kurosaka M, *et al*. 2009. Early flowering phenotype in transgenic pears (*Pyrus communis* L.) expressing the *CiFT* gene. *Journal of the Japanese Society for Horticultural Science* **78**: 410–416.

Michaels SD, Himelblau E, Kim SY, Schomburg FM, Amasino RM. 2005. Integration of flowering signals in winter-annual *Arabidopsis*. *Plant Physiology* **137**: 149–156.

Moss GI. 1971. Effect of fruit on flowering in relation to biennial bearing in sweet orange (*Citrus sinensis*). *Journal of Horticultural Science & Biotechnology* **46**: 177.

Muñoz-Fambuena N, Mesejo C, González-Mas MC, Primo-Millo E, Agustí M, Iglesias DJ. 2011. Fruit regulates seasonal expression of flowering genes in alternate-bearing 'Moncada' mandarin. *Annals of Botany* **108**: 511–519.

Muñoz-Fambuena N, Mesejo C, González-Mas MC, Iglesias J, Primo-Millo E, Agustí M. 2012. Gibberellic acid reduces flowering intensity in sweet orange (*Citrus sinensis* L. Osbeck) by repressing *CiFT* gene expression. *Plant Growth Regulation*; doi:10.1007/s00344-012-9263-y.

Nishikawa F, Endo T, Shimada T, *et al*. 2007. Increased *CiFT* abundance in the stem correlates with floral induction by low temperature in Satsuma mandarin (*Citrus unshiu* Marc.). *Journal of Experimental Botany* **58**: 3915–3927.

Nishikawa F, Endo T, Shimada T, *et al*. 2010. Transcriptional changes in *CiFT*-introduced transgenic trifoliate orange (*Poncirus trifoliata* L. Raf.). *Tree Physiology* **30**: 431–439.

Notaguchi M, Abe M, Kimura T, *et al*. 2008. Long-distance, graft-transmissible action of *Arabidopsis* FLOWERING LOCUS T protein to promote flowering. *Plant and Cell Physiology* **49**: 1645–1658.

Peña L, Martin-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. *Nature Biotechnology* **19**: 263–267.

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

Pillitteri LJ, Lovatt CJ, Walling LL. 2004*b*. Isolation and characterization of *LEAFY* and *APETALA1* homologues from *Citrus sinensis* L. Osbeck 'Washington'. *Journal of the American Society for Horticultural Science* **129**: 846–856.

Rottmann WH, Meilan R, Sheppard LA, *et al*. 2000. Diverse effects of overexpression of *LEAFY* and *PTLF*, a poplar (*Populus*) homolog of *LEAFY/FLORICAULA*, in transgenic poplar and *Arabidopsis*. *Plant Journal* **22**: 235–245.

Schneider H. 1968. The anatomy of *Citrus*. In: Reuther W, Batchelor LD, Webber HJ. eds. *The citrus industry*, Vol. II. Berkeley, CA: University of California, 1–86.

Sherman WG, Beckman TG. 2003. The climatic adaptation in fruit crops. *Acta Horticulturae* **622**: 411–428.

Southerton SG, Strauss SH, Olive MR, *et al*. 1998. *Eucalyptus* has a functional equivalent of the *Arabidopsis* floral meristem identity gene *LEAFY*. *Plant Molecular Biology* **37**: 897–910.

Sreekantan L, Thomas MR. 2006. *VvFT* and *VvMADS8*, the grapevine homologues of the floral integrators *FT* and *SOC1*, have unique expression patterns in grapevine and hasten flowering in *Arabidopsis*. *Functional Plant Biology* **33**: 1129–1139.

Takada S, Goto K. 2003. *TERMINAL FLOWER2*, an *Arabidopsis* homolog of *HETEROCHROMATIN PROTEIN1*, counteracts the activation of *FLOWERING LOCUS T* by *CONSTANS* in the vascular tissues of leaves to regulate flowering time. *Plant Cell* **15**: 2856–2865.

Takada S, Kotaka T, Ohto M, *et al*. 2002. *Arabidopsis* flowering gene *TERMINAL FLOWER2* (*TFL2*) encodes an *HP1*-like protein and negatively regulates *FT* expression. *Plant and Cell Physiology* **43**: pS180.

Tan FC, Swain SM. 2007. Functional characterization of *AP3*, *SOC1* and *WUS* homologues from citrus (*Citrus sinensis*). *Physiologia Plantarum* **131**: 481–495.

Terol J, Naranjo MA, Ollitrault P, Talón M. 2008. Development of genomic resources for *Citrus clementina*: characterization of three deep-coverage BAC libraries and analysis of 46000 BAC end sequences. *BMC Genomics* **9**: 423.

Trankner C, Lehmann S, Hoenicka H, *et al.* 2010. Over-expression of an *FT*-homologous gene of apple induces early flowering in annual and perennial plants. *Planta* **232**: 1309–1324.

Vemmos SN. 1999. Carbohydrate content of inflorescent buds of defruited and fruiting pistachio (*Pistacia vera* L.) branches in relation to biennial bearing. *Journal of Horticultural Science & Biotechnology* **74**: 94–100.

Wada M, Cao QF, Kotoda N, Soejima J, Masuda T. 2002. Apple has two orthologues of *FLORICAULA/LEAFY* involved in flowering. *Plant Molecular Biology* **49**: 567–577.

Wigge PA, Kim MC, Jaeger KE, *et al*. 2005. Integration of spatial and temporal information during floral induction in *Arabidopsis*. *Science* **309**: 1056–1059.

Yahata D, Matsumoto K, Ushijima K. 2004. Relationship between flower-bud differentiation and carbohydrate contents in spring shoots of very-early, early and late maturing cultivars of satsuma mandarin. *Journal of the Japanese Society for Horticultural Science* **73**: 405–410.

Yamaguchi A, Kobayashi Y, Goto K, Abe M, Araki T. 2005. *TWIN SISTER OF FT* (*TSF*) acts as a floral pathway integrator redundantly with *FT*. *Plant and Cell Physiology* **46**: 1175–1189.

Yamamoto S, Abe M, Ichinoki H, Kobayashi Y, Araki T. 2004. Effect of the *Arabidopsis* flowering-time gene *FD* on the expression of meristem identity gene *AP1*. *Plant and Cell Physiology* **45**: pS71.

Yan J, Yuan F, Long G, Qin L, Deng Z. 2012. Selection of reference genes for quantitative real-time RT-PCR analysis in citrus. *Molecular Biology Reports* **39**: 1831–1838.

Yu QY, Moore PH, Albert HH, Roader AHK, Ming R. 2005. Cloning and characterization of a *FLORICAULA/LEAFY* ortholog, *PFL*, in polygamous papaya. *Cell Research* **15**: 576–584.

Yu XH, Klejnot J, Lin CT. 2006. Florigen: one found, more to follow? *Journal of Integrative Plant Biology* **48**: 617–621.