Fruit regulates seasonal expression of flowering genes in 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* The presence of fruit has been widely reported to act as an inhibitor of flowering in fruit trees. This study is an investigation into the effect of fruit load on flowering of 'Moncada' mandarin and on the expression of putative orthologues of genes involved in flowering pathways to provide insight into the molecular mechanisms underlying alternate bearing in citrus.

• *Methods* The relationship between fruit load and flowering intensity was examined first. Defruiting experiments were further conducted to demonstrate the causal effect of fruit removal upon flowering. Finally, the activity of flowering-related genes was investigated to determine the extent to which their seasonal expression is affected by fruit yield.

• *Key Results* First observations and defruiting experiments indicated a significant inverse relationship between preceding fruit load and flowering intensity. Moreover, data indicated that when fruit remained on the tree from November onwards, a dramatic inhibition of flowering occurred the following spring. The study of the expression pattern of flowering-genes of *on* (fully loaded) and *off* (without fruits) trees revealed that homologues of *FLOWERING LOCUS T (FT)*, *SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)*, *APETALA1 (AP1)* and *LEAFY (LFY)* were negatively affected by fruit load. Thus, *CiFT* expression showed a progressive increase in leaves from *off* trees through the study period, the highest differences found from December onwards (10-fold). Whereas differences in the relative expression of *SOC1* only reached significance from September to mid-December, *CsAP1* expression was constantly higher in those trees through the whole study period. Significant variations in *CsLFY* expression only were found in late February (close to 20 %). On the other hand, the expression of the homologues of *TERMINAL FLOWER 1 (TFL1)* and *FLOWERING LOCUS C (FLC)* did not appear to be related to fruit load.

• *Conclusions* These results suggest for the first time that fruit inhibits flowering by repressing *CiFT* and *SOC1* expression in leaves of alternate-bearing citrus. Fruit also reduces *CsAP1* expression in leaves, and the significant increase in leaf *CsLFY* expression from *off* trees in late February was associated with the onset of floral differentiation.

Key words: Alternate bearing, *AP1*, citrus, *FLC*, flowering, fruit load, *FT*, gene expression, *LFY*, *SOC1*, *TFL1*.

INTRODUCTION

Citrus, like many other woody species, flower profusely. In the Mediterranean area, flower induction takes place in autumn/winter and the main blossoming in spring (Sherman and Beckman, 2003). Current knowledge discriminates between exogenous and endogenous components for regulating flowering biology in citrus (Krajewski and Rabe, 1995; Martínez-Fuentes *et al.*, 2004). The former mainly includes climatic factors. Thus, environmental cues such as temperature or photoperiod, and stress conditions (e.g. water deficit or salinity), have been reported as modulators of flowering responses (Agustí, 1999; Valiente and Albrigo, 2004). On the other hand, endogenous factors are essentially genetic and/or hormonal. Phytohormones, such as cytokinins, polyamines or gibberellins, are known to participate to a certain extent in the physiological processes regulating flower induction or differentiation (Koshita *et al.*, 1999). In particular,

gibberellins have been traditionally considered as essential inhibitors of flower bud induction (Mutasa-Gottgens and Hedden, 2009).

In addition, the presence of fruit has also been found to be an inhibitor of flowering in fruit trees. The response of flowering to fruit load has been reported in numerous species (Garner and Lovatt, 2008; Spinelli *et al.*, 2009; Rosenstock *et al.*, 2010), including citrus (Monselise and Goldschmidt, 1982). This behaviour basically consists in the fluctuating production between heavy fruit yields (*on* year) followed by scarce ones (*off* year). Moreover, the inhibitory effect of fruit in flower formation depends on both the number of fruits developed and the harvest date (El-Otmani *et al.*, 2004; Martínez-Fuentes *et al.*, 2010). Hormonal factors, competition for nutrients or even changes in carbohydrate and mineral status (Goldschmidt *et al.*, 1985; Valiente and Albrigo, 2004; Baninasab *et al.*, 2007; Rohla *et al.*, 2007) appear to participate in the regulation processes, although the way in which the presence of fruit

affects flowering and the nature of the regulatory mechanisms remains unknown.

Recently, the development of genomic and transcriptomic tools has contributed to a better understanding of the metabolic and molecular processes involved in floral biology. Most of our knowledge about flower induction has come from studying flowering regulatory genes in *Arabidopsis thaliana* (Komeda, 2004). These genes appear to be extraordinarily conserved in woody species (Brunner and Nilsson, 2004), and previous research has demonstrated that many of them even share roles and/or metabolic pathways (Greenup *et al.*, 2009). Moreover, current evidence assumes that flowering is the result of complex interactions at the metabolic and molecular level involving multiple promoter and inhibitor genes (Moon *et al.*, 2005; Michaels and Michaels, 2009). Regarding citrus, several studies have recently been conducted to eluci- date the molecular mechanisms involved in flower formation and differentiation. A first approach on citrus was developed by Peña *et al.* (2001), who demonstrated through plant trans- formation the role of *APETALA1* (*AP1*) and *LEAFY* (*LFY*) genes in juvenile phase development. Later studies have ident- ified and/or isolated in this species these (*CsAP1*, *CsLFY*; Pillitteri *et al.*, 2004b) and other regulatory genes involved in both the determination of the flowering time and floral iden- tity processes (see Dornelas *et al.*, 2007).

The regulatory role of the *FLOWERING LOCUS T* (*FT*) gene has been identified in numerous species (Turk *et al.*, 2008; Zhang *et al.*, 2010) including citrus (Endo *et al.*, 2005; Nishikawa *et al.*, 2007), and the encoded protein (*FT* protein) associated with the mythic 'florigen' (Yu *et al.*, 2006). Other genes, like *CONSTANS* (*CO*) or *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) have been reported to integrate signals from different pathways and, at least in *Arabidopsis*, are involved in promoting vegeta- tive to reproductive tissues (Borner *et al.*, 2000; Lee *et al.*, 2000; Onouchi *et al.*, 2000; Komeda, 2004). Specifically, *CO* participates in upstream and complex regulatory pathways, whereas SOC1 appears to act downstream (Dornelas *et al.*, 2007). In contrast, *FLOWERING LOCUS C* (*FLC*) represses flowering; high transcript levels have been found in late- flowering mutants, suggesting a central role of this gene in the control of flowering time, mainly through the vernalization pathway (Sheldon *et al.*, 2000; Michaels *et al.*, 2005). Even though other repressor genes of flowering, like *TERMINAL FLOWER 1* (*TFL1*), have been isolated from 'Washington' navel sweet orange (*CsTFL*; Pillitteri *et al.*, 2004a) and corre- lated positively with juvenility, rigorous information about the function of *FLC* in citrus is not yet available.

In summary, alternate bearing is the result of complex meta- bolic and molecular regulatory pathways affecting flowering induction and floral identity. The phenomenon has been largely explained as a crop load dependency and, although the factors modulating the processes involved have recently been studied for diverse species and key genes regulating flow- ering identified, we lack knowledge about the effects of crop load and the molecular mechanisms involved. In this work, for the first time, the effects of fruit on the expression of puta- tive homologues of genes involved in flowering pathways were analysed to provide insight into the molecular mechanisms underlying alternate bearing in citrus due to crop load.

MATERIALS AND METHODS

Plant material

This study involved field grown 12-year-old 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. Experimental fields were located in the IVIA Research Station (Moncada, Spain). Trees of this cultivar exhibit a marked alternate-bearing behaviour.

Fruit load and flowering relationship

To examine the effect of fruit load on flowering, 25 trees were randomly selected for their uniformity in size and vigour at spring. Total yield per tree was determined by count- ing and weighing all fruits at harvest (April), and flowering intensity was evaluated in spring as follows. Four branches per tree of three ages (late spring, summer and autumn sprouts) with some 300 nodes per branch were previously selected. Both the number of sprouted nodes and sprouts were counted. The flowers per sprout were also counted, obtaining the results as the number of flowers per 100 nodes to compensate for the differences in size of the selected branches.

Defruiting experiments were performed on another set of 24 trees using four levels of fruit removal (0, 33 %, 66 % and 100 %). Treatments were performed at the onset of stage II of fruit development (July). A randomized complete-block design was used in the experiments.

From early September to the end of February, 30 fully developed mature adult leaves per tree from *on* (fully loaded) and *off* trees (without fruits) were collected for RNA extractions. Samples were pooled into three groups, and immediately ground and stored at −80 °C until analysed.

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 instructions. RNA quality was tested by 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 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 Diagnostics, Basel, Switzerland) equipped with LightCycler Software version 4·0. One-step RT-PCR was carried out. Reactions con- tained 2·5 units of MultiScribe Reverse Transcriptase (Applied Biosystems, Carlsbad, CA, USA), 1 unit of RNase Inhibitor (Applied Biosystems), 2 μL LC FastStart DNA MasterPLUS SYBRGreen I (Roche Diagnostics, Basel, Switzerland), 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, 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 10-fold dilution series of an RNA sample as a standard curve. Relative mRNA levels were then normalized to total RNA amounts as previously described (Bustin, 2002; Hashimoto *et al.*, 2004) and an expression value of 1 was arbitrarily assigned to the first sample of the *on* trees. 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 *et al.*, 2007). Synthetic oligonucleotides were designed to amplify the gene from the selected clones and, as stated before, sequenced for confirmation. Details about the forward and reverse primers are listed in Table 1.

Statistical analyses

Parameters were statistically tested by analyses of variance (ANOVA), using the least significant differences (LSD) test for means separation. The experimental data were analysed with Statgraphics Plus 5·1 software (Statistical Graphics, Englewood Cliffs, NJ, USA).

RESULTS

Fruit load–flowering relationships

A significant inverse relationship between preceeding fruit load and flowering intensity was found in 'Moncada' mandarin ($r = -0.93$; $P \le 0.05$; $n = 25$; Fig. 1). Considering the results, the larger the crop load the lower the flowering intensity, with a breaking point of about 50 kg tree^{-1}. Above this fruiting value, flowering was independent of crop load and paralleled nearly nil values.

Although statistically significant, this relationship does not imply causality, therefore further experiments were carried out to demonstrate the direct effect of fruit removal upon flowering. As expected, control *on* trees (non-defruited, fully loaded) showed the lowest number of flowers (0·5 flowers/ 100 nodes, on average), in comparison with those completely defruited, which presented the highest number of flowers (142 flowers/100 nodes). Intermediate fruit loads (33 % and 66 % defruited trees) resulted in intermediate flowering intensities (Fig. 2).

The effect of fruit removal on flowering was also evident on fruit set and yield assessment. The higher the flowering intensity, the larger the crop load (data not shown). The magnitude of the response, however, depended on the time of fruit removal. Thus, removal of all fruits in August, September or October did not affect flowering in spring (100–135 flowers/ 100 nodes; Fig. 3); however, when fruit removal was performed from November onwards, a dramatic inhibition of flowering was observed the following spring (<10 flowers/ 100 nodes).

Expression of flowering-related genes

The time-course of *CiFT* expression in leaves throughout the study was strongly affected by fruit load (Fig. 4). Significant differences in mRNA transcripts were detected between *on* and *off* trees from October onwards. The expression in *off* tree leaves increased progressively, becoming more than 10-fold higher than that of *on* tree leaves in December, and remaining almost constant up to late in February. *CiFT* transcripts in *on* tree leaves did not significantly vary within the study period.

The time-course of *SOC1* relative expression in leaves was also higher in *off* trees than in *on* trees (Fig. 5). Differences were statistically significant from early September to mid-December, becoming highest in November (close to 50 % higher for *off* trees). From early January onwards, differences between treatments were not statistically significant.

Figure 6 shows the time-course of *FLC* expression in leaves from *on* and *off* trees. From September to mid-December, no differences in gene expression between treatments were found. In January, however, levels of mRNA transcripts markedly increased in *on* trees. From January until the end of the study, expression in *on* tree leaves remained between 1·6- and 2·8-fold higher than in *off* tree leaves.

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	aCL9055Contig1	CAAAACCAGGTTCCCAACAC ACGAACATACGGGTTCAAGG	139
CsLFY	aC34107C06EF_c	TCTTGATCCAGGTCCAGAACATC TAGTCACCTTGGTTGGGCATT	63
FLC	aCL8484Contig1	CGCGACAAACAGAGTGAAAA TGTCTCGCAATCTCCTGTTG	110
SOC1	aCL2263Contig1	CCTCGTTCAACCGTTACCAT GCAAGCCTTCTCTTGCTTTG	100

* EST code refers to the database entry available in Citrus Functional Genomics Project (CFGP; http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/).
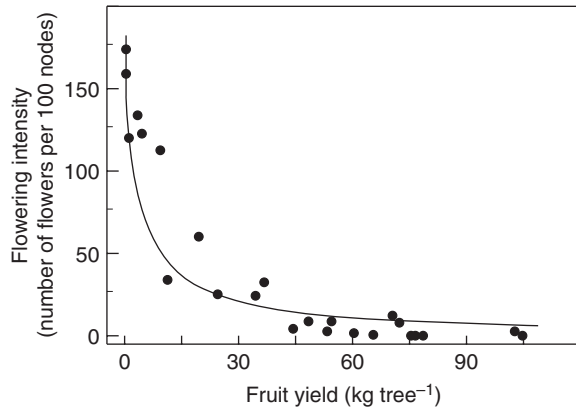
FIG. 1. Fruit load and flowering intensity relationship in 'Moncada' mandarin randomly selected trees growing in the field ($n = 25$). The non-linear regression is significant at $P \leq 0.05$.
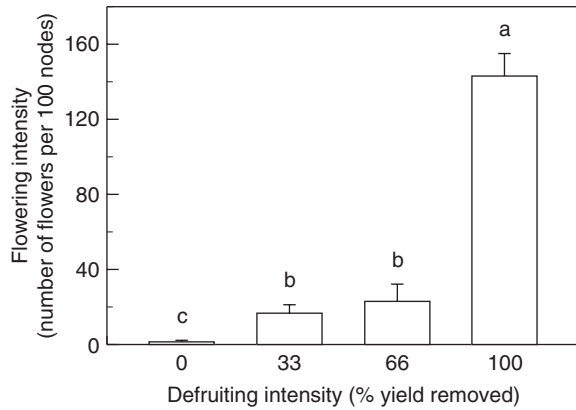

FIG. 2. Effect of defruiting treatments (0, 33, 66 and 100 % of fruit removal) on flowering intensity of 'Moncada' mandarin trees. Fruits were removed in July. Data are the means of six trees per treatment and different letters indicate significant differences ($P \leq 0.05$).
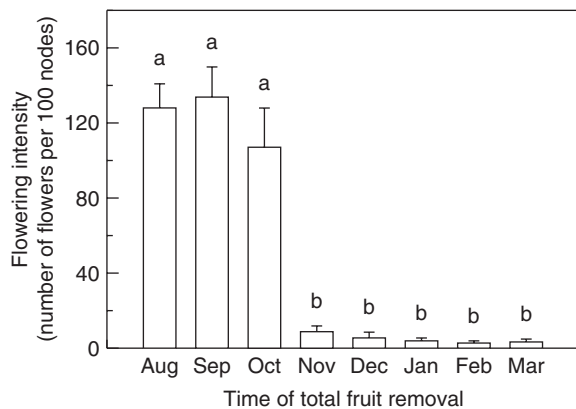

FIG. 3. Effect of fruit removal time on flowering of 'Moncada' mandarin trees. Data are the means of six trees and different letters indicate significant differences ($P \leq 0.05$).
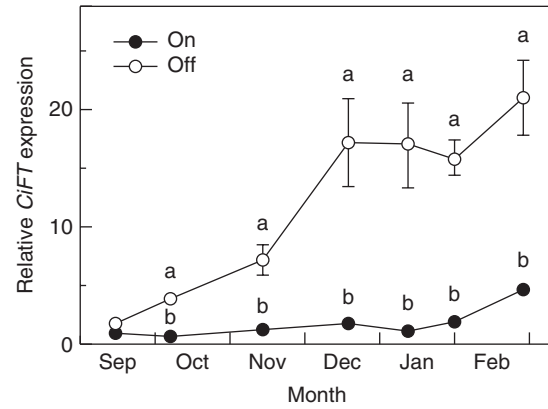

FIG. 4. Time-course of *CiFT* expression in 'Moncada' mandarin leaves from September to February for fully loaded (On) or without-fruit (Off) trees. Data are means ± s.e. of three independent replicates ($n = 3$). Different letters indicate significant differences ($P \leq 0.05$) for each sampling date. Where error bars are not visible they are smaller than the symbol size.

FIG. 5. Time-course of *SOC1* expression in 'Moncada' mandarin leaves from September to February for fully loaded (On) or without-fruit (Off) trees. Data are means ± s.e. of three independent replicates ($n = 3$). Different letters indicate significant differences ($P \leq 0.05$) for each sampling date. Where error bars are not visible they are smaller than the symbol size.
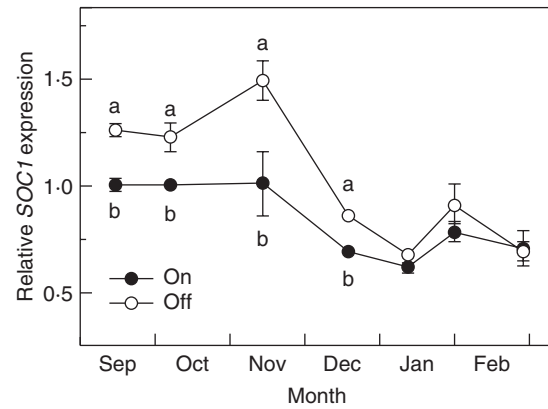

FIG. 6. Time-course of *FLC* expression in 'Moncada' mandarin leaves from September to February for fully loaded (On) or without-fruit (Off) trees. Data are means ± standard error of three independent replicates ($n = 3$). Different letters indicate significant differences ($P \leq 0.05$) for each sampling date. Where error bars are not visible they are smaller than the symbol size.
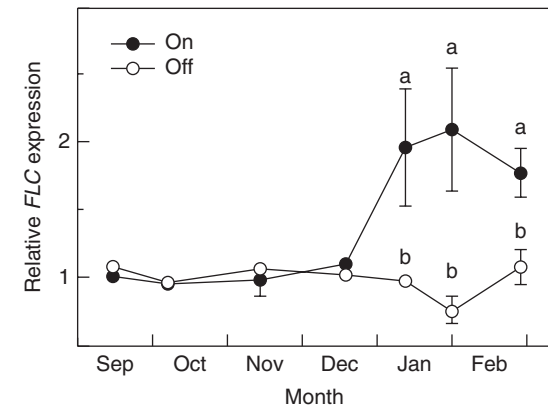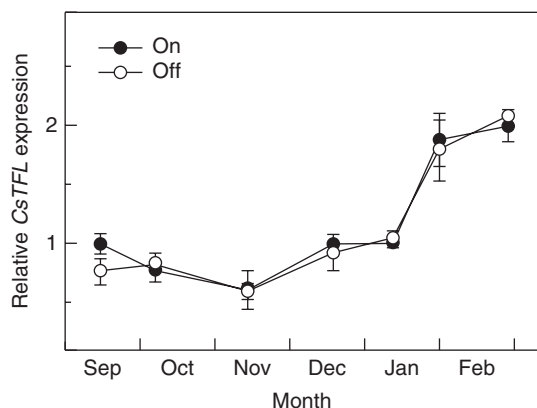
FIG. 7. Time-course of *CsTFL* expression in 'Moncada' mandarin leaves from September to February for fully loaded (On) or without-fruit (Off) trees. Data are means ± s.e. of three independent replicates (*n* = 3). Different letters indicate significant differences (*P* ≤ 0·05) for each sampling date. Where error bars are not visible they are smaller than the symbol size.
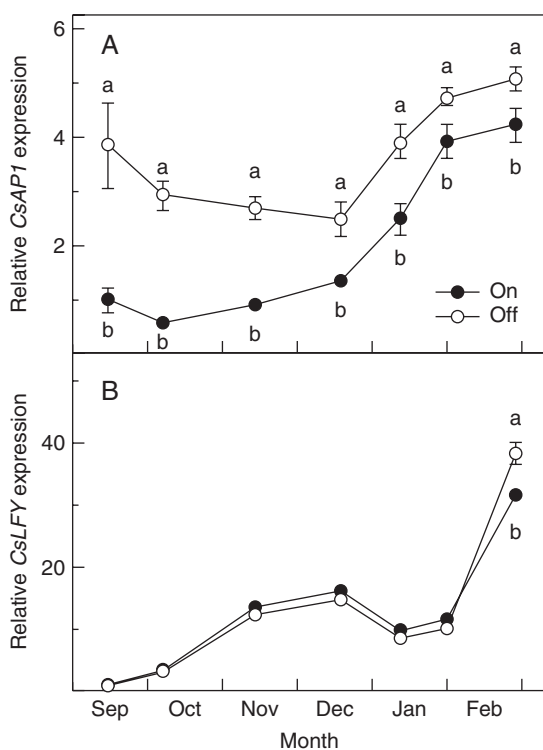

FIG. 8. Accumulation of *CsAP1* (A) and *CsLFY* (B) transcripts in 'Moncada' mandarin leaves from September to February for fully loaded (On) or without-fruit (Off) trees. Data are means ± s.e. of three independent replicates (*n* = 3). Different letters indicate significant differences (*P* ≤ 0·05) for each sampling date. Where error bars are not visible they are smaller than the symbol size.

Expression of *CsTFL* in leaves was slightly reduced from September to mid-November; afterwards, it continuously increased up to the end of February, when it was >3 times the amount measured in November (Fig. 7). No significant differences were found between leaves from *on* and *off* trees during the study period.

CsAP1 and *CsLFY* revealed different patterns of change in response to fruit load (Fig. 8). *CsAP1* expression remained almost constant from early October up to the middle of December. During this period, expression values were 3–5 times lower in *on* trees than in *off* trees. From December onwards expression increased in both cases and, although minor in magnitude, significant differences between *on* and *off* trees were also found until the end of February. On the other hand, *CsLFY* expression progressively increased from September to the middle of December (15- to 20-fold). After a transitory reduction in January, expression considerably augmented towards the end of February, reaching values between 30 and 50 times those registered in September. Differences between *on* and *off* trees were only significant at the end of the study. In late February, *CsLFY* expression in *off* trees was significantly higher than that found in *on* trees (close to 20 %).

DISCUSSION

Alternate bearing has been associated primarily with the presence of fruit (Verreynne and Lovatt, 2009; Martínez-Fuentes *et al.*, 2010). According to the literature, it is assumed that crop load alters both flower induction and floral bud differentiation (Valiente and Albrigo, 2004). Particularly, in citrus, flower induction has been reported to occur late in autumn, whereas differentiation occurs afterwards (Iglesias *et al.*, 2007). Recent molecular approaches support this scheme, distinguishing between genes regulating flowering induction and those regulating floral differentiation processes (Tadeo *et al.*, 2008). The present results indicate that genes regulating flower induction are strongly related to fruit load in alternate-bearing citrus trees.

Fruit load acts as a strong inhibitor of flowering in numerous woody fruit tree species including citrus, although the responses vary among species and cultivars (Monselise and Goldschmidt, 1982; Valiente and Albrigo, 2004; Albrigo and Galán, 2004). The present data obtained for 'Moncada' mandarin confirmed, once again, a non-linear fruit load–flowering intensity relationship (Fig. 1). However, since this relationship does not necessarily imply causality, convincing evidence that fruit load is directly responsible for reducing flowering intensity was provided by means of defruiting treatments. Trees maintaining all developed fruits up to maturation hardly flowered (5 flowers/100 nodes, on average), whereas those fully defruited at the onset of stage II of fruit development flowered profusely (142 flowers/100 nodes, on average). Trees defruited at an intermediate intensity (33 % and 66 % of developing fruitlets) flowered intermediately as well. However, because no differences were found between 33 % and 66 % of fruit removal (16 and 22 flowers/100 nodes, respectively) the effect does not appear to be strictly quantitative. These results support the hypothesis that the fruit load–flowering intensity relationship is not a linear function and this coincides with previous reports suggesting that there might be a threshold value for crop load, dependent on the variety and physiological status, above which flowering is strongly inhibited (Agustí *et al.*, 1992; Martínez-Fuentes *et al.*, 2010).

It has been previously reported that flower inhibition due to crop load also depends on the length of time the fruit remains on the tree (see review by Monselise and Goldschmidt, 1982). Considering the present data for 'Moncada' mandarin, fruit

remaining on the tree did not affect return bloom (100–120 flowers/100 nodes) up to November; however, fruit remaining on the tree from November onwards dramatically reduced flowering (values close to 0). Therefore, effective inhibition time of fruit load began between October and November. This period has been associated with the fruit reaching its maximum size (Martínez-Fuentes *et al.*, 2010), or with peel ripening (García-Luis *et al.*, 1986). In any event, fruit drastically inhibits flowering from November onwards, suggesting that there is a point of no return for induction of quiescent buds, and that some irreversible physiological, metabolic or molecular events induced by presence of fruit must be responsible for inhibition. This hypothesis is supported by the fact that bud sensitivity to gibberellic acid (GA$_3$) inhibiting flowering occurs at this moment (García-Luis *et al.*, 1986).

The mechanism whereby fruit load affects flowering intensity is not completely understood, although several regulatory factors have been described. Early observations linked carbohydrate and nitrogen metabolism to the process (Goldschmidt *et al.*, 1985; Lovatt *et al.*, 1988); however, recent studies demonstrated that carbohydrate or nitrogen status are involved in nutritional or storage adjustments rather than in the floral process directly (Reig *et al.*, 2006; Martínez-Fuentes *et al.*, 2010). Several studies have linked flowering intensity to bud sprouting, showing that changes in flowering intensity paralleled changes in the summer/autumn shoot number (Verreynne and Lovatt, 2009; Martínez-Fuentes *et al.*, 2010), and also illustrated that the higher the fruit load the lower the number of sprouted nodes in spring (Martínez-Fuentes *et al.*, 2010). Additionally, environmental factors can modulate flowering through modifications in the physiology of shoot development or even in key metabolic pathways (Agustí, 1999). Nonetheless, knowledge about factors affecting flowering has considerably increased, but data do not provide enough information to understand the mechanisms through which the fruit controls the process of flowering.

Recently, molecular and genomic approaches have been considered important tools to shed light on the complex physiological and metabolic pathways leading to flowering. In recent years, numerous flowering-gene promoters and inhibitors have been identified, isolated and characterized. Extensive research has been done in *A. thaliana*, for which the balance between promoters and inhibitors is decisive for the adequate determination of flowering time and floral identity (Chon and Yang, 1999; Kobayashi *et al.*, 1999). However, in woody tree species, little has been done on this subject. The results presented herein regarding the expression of flowering genes affected by fruit load offer an insight into the molecular mechanisms underlying alternate bearing in citrus trees.

In several species, flowering ability has been demonstrated to be influenced by the integration of environmental signals from the photoperiod and vernalization pathways (Onouchi *et al.*, 2000; Amasino, 2005; Sheldon *et al.*, 2009), mainly modulated by two floral integrators, the *FT* and the *SOC1* genes (Kardailsky *et al.*, 1999; Borner *et al.*, 2000; Lee *et al.*, 2000; Samach *et al.*, 2000). Both genes have been described as floral promoters and their overexpression induces early-flowering phenotypes (Lee *et al.*, 2006; Sreekantan and Thomas, 2006; Zhang *et al.*, 2010).

The *FT* gene has been demonstrated to be a pivotal factor controlling flowering period in numerous species (Faure *et al.*, 2007; Chab *et al.*, 2008; Hisamoto *et al.*, 2008; Zhang *et al.*, 2010). The present results support this hypothesis and further relate it to the effects of fruit load in citrus trees just as autumn/winter temperature enhances its expression (Nishikawa *et al.*, 2007). Leaves from *off* trees (high-return bloom-flowering in spring) showed significantly increased mRNA transcript levels as compared with those from *on* trees (low return bloom). Thus, whereas *on* trees showed stationary basal levels of expression, a progressive increase in *CiFT* levels was observed in leaves from *off* trees (Fig. 4) until December, concomitantly with a higher flowering rate the following spring (Fig. 2). These results demonstrate, on the one hand, that an increased FT protein constitutes a signal *per se* that exports from leaf to the shoot apical meristem, where floral differentiation takes place (Notaguchi *et al.*, 2008) and, on the other, that a translated FT protein can be translocated to the floral meristem at any time (Lin *et al.*, 2007; Turck *et al.*, 2008). Nevertheless, the translocation pathway followed by FT protein into the apex remains unknown.

Although not always significant, *SOC1* also showed higher expression levels in leaves from *off* trees compared with those registered for *on* trees, particularly during the floral induction period (Fig. 5). Lee and Lee (2010) reported that constitutive expression of *SOC1* promotes early flowering, while recessive loss-of-function seems to delay flowering (Onouchi *et al.*, 2000). Although isolated in only a few plant species, highly conserved homologues of this gene have been identified in numerous species, including citrus, in which the constitutive expression of several *SOC1-like* homologues induces early flowering and delays senescence of floral organs (Tan and Swain, 2007). In this sense, the present results confirm the role of this gene in flowering resulting from alternate-bearing adult trees.

Unlike the functions attributed to *SOC1* and *FT* genes, *FLC* has been described to encode a MADS-domain protein able to repress flowering (Michaels and Amasino, 1999). The present data showed no differences in *FLC* activity between *on* and *off* trees until December (Fig. 6). From this time onwards, there was a progressive increase in transcript levels in leaves from *on* trees, whereas those from *off* trees showed no change or even a slight decrease. Some authors have pointed out that vernalization promotes flowering through a permanent epigenetic repression of *FLC* (Michaels and Amasino, 1999), probably through histone methylation and changes in chromatin conformation (Bastow *et al.*, 2004; Sung and Amasino, 2004). In the present study, trees with increased amounts of *FLC* mRNA transcripts near spring corresponded to *on* trees that did not flower. Additionally, previous reports have indicated antagonistic effects of flowering promoters and *FLC* on the expression of target genes leading to flowering. The study of regulatory pathways demonstrates that this gene is located upstream along the major pathways, since *FT* overexpression does not appear to affect *FLC* expression (Moon *et al.*, 2005; Lee and Lee, 2010). Likewise, it has been proposed that elevated levels of *FLC* expression may be responsible for reductions in *FT* activity (Michaels *et al.*, 2005). In this context, the marked increase in *FLC* measured in the *on* trees from December onwards (Fig. 6) might be related to the suppression

of *CiFT* activity in their leaves compared with *off* trees (Fig. 4) and, therefore, with the inhibition of flowering.

Moreover, the results show that *SOC1* expression increased in *off* trees before the sharp increase in *CiFT*, so the activation pathways appear to work, at least partially, autonomously; furthermore, from November onwards, *SOC1* expression did not parallel the increased *CiFT* gene expression. This is not unusual since, at least partially, self-determining regulatory mechanisms for *FT* and *SOC1* activity have been reported recently (Lee and Lee, 2010). Therefore, this hypothesis can be extrapolated to the molecular mechanisms affecting flowering through effects of fruit load. Moreover, whereas *CiFT* expression was significantly higher in *off* trees as compared with *on* trees until the end of February, significant differences in *SOC1* expression disappeared by the beginning of January.

Although *TFL1* has been described as a crucial floral timing regulator in several species (Shannon and Meeks-Wagner, 1991; Liljegren *et al.*, 1999), in the present study no differences in *CsTFL* expression dependent on fruit load were found (Fig. 7). This gene was identified and isolated in *Citrus sinensis* (Pillitteri *et al.*, 2004*a*) and has been proposed to participate in the development and maintenance of vegetative growth, repressing flowering in several plant species (Esumi *et al.*, 2010; Mohamed *et al.*, 2010). In addition Nishikawa *et al.* (2009) have reported no variations in the expression of this gene during induction and flower bud development, neither in trifoliate and deciduous *Citrus*-like species (*P. trifoliata*) nor in Satsuma mandarin (*C. unshiu*), indicating that this gene is related to juvenility processes rather than to annual floral transition. Since there were no differences between *on* and *off* trees regarding *CsTFL* expression, differences in flowering as a consequence of fruit load do not appear to be dependent on this gene.

Finally, *AP1* and *LFY* genes have been reported to promote flowering in diverse species including citrus, although their primary role appears to be linked to the determination of floral identity (Pillitteri *et al.*, 2004*b*). Although transgenic approaches have demonstrated that both genes strongly interact with other physiological processes, such as competition for nutrients, their constitutive expression *per se* reduces the juvenility period in transgenic citrus (Peña *et al.*, 2001; Duan *et al.*, 2010). In particular, *AP1* seems to be more effective and dynamic than *LFY* in the induction of flowering (Peña *et al.*, 2001), orchestrating floral initiation by repressing the action of inhibitors at the level of the meristem as stated by Kaufmann *et al.* (2010). The present results showed a reduced expression of *CsAP1* in leaves in response to the presence of fruit (Fig. 8). Differences between the levels of expression in leaves from *on* and *off* trees were significant during the whole study period despite the reduction in these levels from January onwards. It is noteworthy that *LFY* activity has been associated with the control of floral meristem identity in *Arabidopsis* (Weigel *et al.*, 1992). Moreover, recent studies on fruit trees, including citrus, demonstrated that once floral identity is determined, high *LFY* expression levels are almost limited to reproductive organs (Pillitteri *et al.*, 2004*b*). This observation might also be linked to the absence of differences in *CsLFY* expression in leaves from *on* and *off* trees between September and January. The significantly higher values found in *off* trees at the end of February support the main

role of this gene on bud differentiation and/or floral identity rather than on inductive processes.

In conclusion, in *Citrus* fruit load inhibits flowering by repressing *CiFT* and *SOC1* expression in leaves during the floral bud induction period, whereas *CsTFL* and *FLC* expression does not seem to be associated with fruit load. Fruit load reduces *CsAP1* gene expression in leaves during the floral bud induction period, although differences between *on* and *off* trees were attenuated from January onwards, indicating the onset of floral differentiation. This hypothesis is also reinforced by the significant increase in *CsLFY* expression found in leaves from *off* trees only in late February. The *FLC*-increased expression in *on* trees from early winter onwards might well be related to the suppression of *CiFT* expression in their leaves and, therefore, with inhibition of flowering.

LITERATURE CITED

Agustí M. 1999. Preharvest factors affecting postharvest quality of citrus fruit. In: Schirra M. ed. *Advances in postharvest diseases and disorders control of citrus fruit*. Trivandrum, India: Research Signpost, 1–34.

Agustí M, Almela V, Pons J. 1992. Effects of girdling on alternate bearing in citrus. *Journal of Horticultural Science* **67**: 203–210.

Albrigo LG, Galán V. 2004. Flower bud induction, flowering and fruit-set of some tropical and subtropical fruit tree crops with special reference to citrus. In: Albrigo LG, Galán V. eds. *Proceedings of the 26th International Horticultural Congress: Citrus and Other Subtropical and Tropical Fruit Crops*, 81–91.

Amasino RM. 2005. Vernalization and flowering time. *Current Opinion in Biotechnology* **16**: 154–158.

Baninasab B, Rahemi M, Shariatmadari H. 2007. Seasonal changes in mineral content of different organs in the alternate bearing of pistachio trees. *Communications in Soil Science and Plant Analysis* **38**: 241–258.

Bastow R, Mylne JS, Lister C, Lippman Z, Martienssen RA, Dean C. 2004. Vernalization requires epigenetic silencing of *FLC* by histone methylation. *Nature* **427**: 164–167.

Borner R, Kampmann G, Chandler J, et a**l. 2000.** A MADS domain gene involved in the transition to flowering in *Arabidopsis*. *The Plant Journal* **24**: 591–599.

Brunner AM, Nilsson O. 2004. Revisiting tree maturation and floral initiation in the poplar functional genomics era. *New Phytologist* **164**: 43–51.

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

Chab D, Kolar J, Olson MS, Storchova H. 2008. Two *FLOWERING LOCUS T (FT)* homologs in *Chenopodium rubrum* differ in expression patterns. *Planta* **228**: 929–940.

Chon ML, Yang CH. 1999. Late-flowering genes interact with early-flowering genes to regulate flowering time in *Arabidopsis thaliana*. *Plant and Cell Physiology* **40**: 702–708.

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

Duan YX, Fan J, Guo WW. 2010. Regeneration and characterization of transgenic kumquat plants containing the *Arabidopsis APETALA1* gene. *Plant Cell Tissue and Organ Culture* **100**: 273–281.

El-Otmani M, Goumari M, Srairi I, Lbrek A, Charif L, Lovatt CJ. 2004. Heavy fruit load and late harvest inhibit flowering of the 'Nour' Clementine mandarin. *Proceedings of the International Society of Citriculture* **2**: 525–527.

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, 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.

Faure S, Higgins J, Turner A, Laurie DA. 2007. The *FLOWERING LOCUS T-like* gene family in barley (*Hordeum vulgare*). *Genetics* **176**: 599–609.

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*. *Physiologia Plantarum* **66**: 515–520.

Garner LC, Lovatt CJ. 2008. The relationship between flower and fruit abscission and alternate bearing of 'Hass' avocado. *Journal of the American Society for Horticultural Science* **133**: 3–10.

Goldschmidt EE, Aschkenazi N, Herzano Y, Schaffer AA, Monselise SP. 1985. A role for carbohydrate-levels in the control of flowering in citrus. *Scientia Horticulturae* **26**: 159–166.

Greenup A, Peacock WJ, Dennis ES, Trevaskis B. 2009. The molecular biology of seasonal flowering-responses in *Arabidopsis* and the cereals. *Annals of Botany* **103**: 1165–1172.

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.

Hisamoto Y, Kashiwagi H, Kobayashi M. 2008. Use of flowering gene *FLOWERING LOCUS T (FT)* homologs in the phylogenetic analysis of bambusoid and early diverging grasses. *Journal of Plant Research* **121**: 451–461.

Iglesias DJ, Cercós M, Colmenero-Flores JM, et al. 2007. Physiology of citrus fruiting. *Brazilian Journal of Plant Physiology* **19**: 333–362.

Kardailsky I, Shukla VK, Ahn JH, et al. 1999. Activation tagging of the floral inducer *FT*. *Science* **286**: 1962–1965.

Kaufmann K, Wellmer F, Muino JM, et al. 2010. Orchestration of floral initiation by *APETALA1*. *Science* **328**: 85–89.

Kobayashi Y, Kaya H, Goto K, Iwabuchi M, Araki T. 1999. A pair of related genes with antagonistic roles in mediating flowering signals. *Science* **286**: 1960–1962.

Komeda Y. 2004. Genetic regulation of time to flower in *Arabidopsis thaliana*. *Annual Review of Plant Biology* **55**: 521–535.

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.

Krajewski AJ, Rabe E. 1995. Citrus flowering: a critical evaluation. *Journal of Horticultural Science* **70**: 357–374.

Lee J, Lee I. 2010. Regulation and function of *SOC1*, a flowering pathway integrator. *Journal of Experimental Botany* **61**: 2247–2254.

Lee JH, Hong SM, Yoo SJ, Park OK, Lee JS, Ahn JH. 2006. Integration of floral inductive signals by *FLOWERING LOCUS T* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*. *Physiologia Plantarum* **126**: 475–483.

Lee H, Suh SS, Park E, et al. 2000. The *AGAMOUS-LIKE20 MADS* domain protein integrates floral inductive pathways in *Arabidopsis*. *Genes & Development* **14**: 2366–2376.

Liljegren SJ, Gustafson-Brow A, Pinyopich A, Ditta GS, Yanofsky MF. 1999. Interactions among *APETALA1*, *LEAFY* and *TERMINAL FLOWER 1* specify meristem fate. *The Plant Cell* **11**: 1007–1018.

Lin MK, Belanger H, Lee YJ, et al. 2007. *FLOWERING LOCUS T* protein may act as the long-distance florigenic signal in the cucurbits. *The Plant Cell* **19**: 1488–1506.

Lovatt CJ, Zheng Y, Hake KD. 1988. Demonstration of a change in nitrogen metabolism influencing flower initiation in *Citrus*. *Israel Journal of Botany* **37**: 181–188.

Martínez-Fuentes A, Mesejo C, Juan M, Almela V, Agustí M. 2004. Restrictions on the exogenous control of flowering in citrus. *Acta Horticulturae* **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). *Journal of the Science of Food and Agriculture* **90**: 1936–1943.

Michaels SD, Amasino RM. 1999. *FLOWERING LOCUS C* encodes a novel *MADS* domain protein that acts as a repressor of flowering. *The Plant Cell* **11**: 949–956.

Michaels SD, Michaels AF. 2009. Flowering time regulation produces much fruit. *Current Opinion in Plant Biology* **12**: 75–80.

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

Mohamed R, Wang CT, Ma C, et al. 2010. Populus *CEN/TFL1* regulates first onset of flowering, axillary meristem identity and dormancy release in *Populus*. *The Plant Journal* **62**: 674–688.

Monselise SP, Goldschmidt EE. 1982. Alternating bearing in fruit trees. *Horticultural Reviews* **4**: 128–173.

Moon J, Lee H, Kim M, Lee I. 2005. Analysis of flowering pathway integrators in *Arabidopsis*. *Plant and Cell Physiology* **46**: 292–299.

Mutasa-Gottgens E, Hedden P. 2009. Gibberellin as a factor in floral regulatory networks. *Journal of Experimental Botany* **60**: 1979–1989.

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, Fujii H, Shimizu T, Omura M. 2009. Differences in seasonal expression of flowering genes between deciduous trifoliate orange and evergreen Satsuma mandarin. *Tree Physiology* **29**: 921–926.

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.

Onouchi H, Igeno MI, Perilleux C, Graves K, Coupland G. 2000. Mutagenesis of plants overexpressing *CONSTANS* demonstrates novel interactions among *Arabidopsis* flowering-time genes. *The Plant Cell* **12**: 885–900.

Peña L, Martin-Trillo M, Juarez J, Pina JA, Navarro L, Martinez-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. 2004a. 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. 2004b. 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.

Reig C, Gonzalez-Rossia D, Juan M, Agustí M. 2006. Effects of fruit load on flower bud initiation and development in peach. *Journal of Horticultural Science & Biotechnology* **81**: 1079–1085.

Rohla CT, Smith MW, Maness NO, Reid W. 2007. Comparison of return bloom and nonstructural carbohydrates, nitrogen, and potassium concentrations in moderate and severe alternate-bearing pecan cultivars. *Journal of the American Society for Horticultural Science* **132**: 172–177.

Rosenstock TS, Rosa UA, Plant RE, Brown PH. 2010. A reevaluation of alternate bearing in pistachio. *Scientia Horticulturae* **124**: 149–152.

Samach A, Onouchi H, Gold SE, et al. 2000. Distinct roles of *CONSTANS* target genes in reproductive development of *Arabidopsis*. *Science* **288**: 1613–1616.

Shannon S, Meeks-Wagner DR. 1991. A mutation in the *Arabidopsis TFL1* gene affects inflorescence meristem development. *The Plant Cell* **3**: 877–892.

Sheldon CC, Finnegan EJ, Peacock WJ, Dennis ES. 2009. Mechanisms of gene repression by vernalization in *Arabidopsis*. *The Plant Journal* **59**: 488–498.

Sheldon CC, Rouse DT, Finnegan EJ, Peacock WJ, Dennis ES. 2000. The molecular basis of vernalization: The central role of *FLOWERING LOCUS C (FLC)*. *Proceedings of the National Academy of Sciences of the USA* **97**: 3753–3758.

Sherman WB, Beckman TG. 2003. Climatic adaptation in fruit crops. *Acta Horticulturae* **622**: 411–428.

Spinelli F, Fiori G, Noferini M, Sprocatti M, Costa G. 2009. Perspectives on the use of a seaweed extract to moderate the negative effects of alternate

bearing in apple trees. *Journal of Horticultural Science & Biotechnology* **84**: 131–137.

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.

Sung SB, Amasino RM. 2004. Vernalization and epigenetics: how plants remember winter. *Current Opinion in Plant Biology* **7**: 4–10.

Tadeo FR, Cercós M, Colmenero-Flores JM, et al**. 2008.** Molecular physiology of development and quality of citrus. *Advances in Botanical Research* **47**: 147–223.

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

Terol J, Conesa A, Colmenero JM, et al**. 2007.** Analysis of 13000 unique *Citrus* clusters associated with fruit quality, production and salinity tolerance. *BMC Genomics* **8**: 31. doi:10.1186/1471-2164-8-31.

Turck F, Fornara F, Coupland G. 2008. Regulation and identity of florigen: *FLOWERING LOCUS T* moves center stage. *Annual Review of Plant Biology* **59**: 573–594.

Valiente JI, Albrigo LG. 2004. Flower bud induction of sweet orange trees (*Citrus sinensis* L. Osbeck): effect of low temperatures, crop load, and bud age. *Journal of the American Society for Horticultural Science* **129**: 158–164.

Verreynne JS, Lovatt CJ. 2009. The effect of crop load on budbreak influences return bloom in alternate bearing 'Pixie' mandarin. *Journal of the American Society for Horticultural Science* **134**: 299–307.

Weigel D, Alvarez J, Smyth DR, Yanofsky MF, Meyerowitz EM. 1992. *LEAFY* controls floral meristem identity in *Arabidopsis*. *Cell* **69**: 843–859.

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

Zhang HL, Harry DE, Ma C, et al**. 2010.** Precocious flowering in trees: the *FLOWERING LOCUS T* gene as a research and breeding tool in *Populus*. *Journal of Experimental Botany* **61**: 2549–2560.