Original article

Genetic inhibition of flowering differs between juvenile and adult *Citrus* trees

Muñoz-Fambuena N[1], Nicolás-Almansa M[2], Martínez-Fuentes A[1], Reig C[1], Iglesias DJ[3], Primo-Millo E[3], Mesejo C[1]* and Agustí, M[1].

[1]Instituto Agroforestal Mediterraneo, Universitat Politècnica de València, Camino de Vera s/n, CP 46022, Valencia, Spain. [2]CEBAS-CSIC, Campus Universitario de Espinardo, CP 30100 Murcia, Spain. [3]Instituto Valenciano de Investigaciones Agrarias, crta Moncada-Náquera, km 4.5, Moncada, CP 46113, Spain.

Running title

Flowering genes inhibition in juvenile and adult citrus trees

For correspondence. E-mail carmeco@upv.es

- **Background & aims** In woody species, the juvenile period maintains the axillary meristems in a vegetative stage, unable to flower, for several years. However, in adult trees, some one-year-old meristems flower whereas others remain vegetative to ensure polycarpic growth habit. Both types of trees, therefore, have not-flowering meristems, and we hypothesize that the molecular mechanism regulating flower inhibition in juvenile trees is different from that of adult trees.
- **Methods** In *Citrus* adult trees, the main endogenous factor inhibiting flower induction is the growing fruit. Thus, we studied the expression of the main flowering time, identity and patterning genes of trees with heavy fruit load (not-flowering adult trees) compared to that of 0.5-year-old trees (not-flowering juvenile trees). Adult trees without fruits (flowering trees) were used as control. Second, we studied the expression of the same genes in the meristems of 0.5, 1, 3, 5, and 7 year-old juvenile trees compared to 10 year-old flowering trees.
- **Key Results** The axillary meristems of juvenile trees are unable to transcribe flowering time and patterning genes during the inductive period, although they are able to transcribe *FLOWERING LOCUS T* citrus ortholog (*CiFT2*) in the leaves. On the other hand, meristems of not-flowering adult trees are able to transcribe the flowering network genes although fail to achieve the transcription threshold required to flower, due to *CiFT2* repression by the fruit. Juvenile meristems progressively achieve gene expression, with age-dependent differences from 0.5 to 7 years, *FD-like* and *CsLFY* being the latest.
- **Conclusions** We conclude that during the juvenile period the inhibitory mechanism of flowering is determined in the immature bud, so that it progressively acquires the flowering ability at the gene expression level of the

flowering-time program, whereas in the adult tree it is determined in the leaf, where repression of the *CiFT2* gene expression occurs.

Keywords Alternate bearing, *AP1*, *Citrus*, *FLC*, flowering, fruit, *FT*, *FD*, juvenility, *LFY*, *TFL1*

INTRODUCTION

Flowering involves the transition of the meristem from the vegetative to the reproductive stage. In fruit trees, this process is impeded, at least, in two phases: (i) during the *juvenile period*, in which the meristem is unable to flower during several years even under floral bud inductive conditions (Albani and Coupland, 2010; Sgamma *et al*., 2014), and (ii) during the *adult period*, when fruit inhibits the ability of buds to differentiate into flowers (Martínez-Fuentes *et al*., 2010). In both cases, the bud meristem remains dormant or grows only vegetatively. However, there is a *paradox* regarding the age of the bud meristem and its ability to flower. Juvenile trees grow and branch during several years from the apical and lateral meristems, respectively (Davies and Albrigo, 1994), giving rise to meristems from one to several year-olds unable to flower, i.e. in an *adult vegetative stage* (Baürle and Dean, 2006). In the case of adult trees, the inhibitory effect of fruit on flowering induction only occurs in one season, but subsequently, in the next one, the new emerging one-year-old meristems are able to flower. Therefore, in juvenile trees all the meristems are unable to flower (regardless the age), whereas in the adult trees a proportion of the current one-year-old meristems can develop into flowers, while other meristems remain in a resting state to ensure a polycarpic growth habit (Albani and Coupland, 2010).

In the model plant *Arabidopsis thaliana*, five genetic pathways explain the transition of meristems from the vegetative to the reproductive stage.They involve the interaction between a set of well-known key genes, such as the flowering time genes *FLOWERING LOCUS T* (*FT*), *FLOWERING LOCUS D (FD)* and *SUPRESSOR OF THE OVEREXPRESSION OF CONSTANS 1* (*SOC1*), the meristem identity genes *LEAFY* (*LFY*) and *APETALLA1* (*AP1*) and the floral patterning genes *SEPALLATA* (*SEP*) and *FRUITFUL* (*FUL*) (see reviews by Albani and Coupland., 2010, and Blümel *et al*., 2015). In brief, flowering is mediated by the autonomous or induced up-regulation of the *FT* gene expression in the leaf, and the resulting protein moves to the meristem where it modifies the flower-identity gene expression through interaction with the *FD* transcription factor (Abe *et al*., 2005). However, there are repressor genes that can inhibit flowering even under exogenous inductive conditions. For instance, the *FT* transcription is repressed by *TEMPRANILLO1* (*TEM1*), a gene that regulates juvenility in plants (Sgamma *et al*., 2014), or by *FLOWERING LOCUS C* (*FLC*), a gene that plays a central role in repressing flowering in the vernalization pathway (Seo *et al*., 2009). In the meristem, *TERMINAL FLOWER1* (*TFL1*) forms an heterodimer *TFL1*/*FD* which represses transcription of FD target genes (Sohn *et al.,* 2007), delays flowering, and prevents upregulation of floral identity genes within the shoot apical meristem to maintain shoot indeterminacy (Hanano and Goto, 2011).

In *Citrus*, an evergreen tree species, flowering is triggered in adult trees either by low temperatures during the cold autumn-winter rest period (which varies dramatically from deciduous fruit tree secies like apple, pear, plum, peach, etc.) or by water stress, that upregulate the expression of the citrus orthologs *CiFT2, CsLFY* and *CsAP1* genes (Nishikawa *et al*., 2007; Chica and Albrigo, 2013), and the orthologs of *SOC1*, *CsSL1* and *CsSL2* (Tan and Swain, 2007). Nevertheless, little is known about the endogenous

mechanisms repressing them. The fruits produce an unknown signal (s) that inhibits floral bud induction, even under exogenous inductive conditions, from the time the fruit is close to complete growth late in summer. The process has been observed in *C. sinensis* (sweet orange) (Martínez-Fuentes *et al.,* 2010) and *C. clementina* (mandarin) (Muñoz-Fambuena *et al.,* 2011) in Mediterranean Climate, and in *C. paradisi* (grapefruit) in Tropical Climate (Betancourt *et al*., 2014). The fruit represses the expression of the *CiFT2* in the leaves, and the meristem-identity genes *CsLFY* and *CsAP1* in the buds (Muñoz-Fambuena et al., 2011; 2012; Shalom *et al*., 2012). Besides, fruit removal induces *CiFT2* and *CsLFY* upregulation in citrus (Shalom *et al*., 2014) and apple (Haberman et al., 2016), and also *TFL1* downregulation in apple (Haberman et al., 2016).

In juvenile citrus trees, early approaches studied the constitutive expression of the *A. thaliana LFY* and *AP1* genes in transgenic seedlings, reducing flowering time from several years to 12-20 months (Peña *et al*., 2001). Similarly, the constitutive overexpression of *CiFT2* induced flowering within 3-22 months (Endo *et al*., 2005). Juvenile wild-type trees are not responsive to low temperatures in terms of *CiFT2* transcription in leaves and stems, and they accumulate a higher level of transcripts of *CsTFL1*, compared to adult flowering trees (Pillitteri *et al*. 2004; Nishikawa *et al*., 2007).

A comprehensive study including the expression of flowering time, meristem identity, and flower patterning genes in woody crops is missing because it is difficult to obtain nonflowering tree mutants. To our knowledge, experiments conducted to study flowering repression in trees compared (i) nonflowering seedlings and adult trees competent to flower (Pilliteri *et al*., 2004; Nishikawa *et al*., 2007; Castillo *et al*., 2013; Sgamma *et al*., 2014), and (ii) ‘on’ trees (nonflowering phenotype) and ‘off’ trees

(flowering phenotype) (Muñoz-Fambuena *et al*., 2011; 2012; Shalom *et al*., 2012; Habermar *et al*., 2016). Thus, we designed a different study which compares the time-course expression of flowering genes in nonflowering seedlings (juveline trees) and adult nonflowering trees ('on' trees) during the floral bud inductive period. We hypothesize that transcription of flowering genes is hampered in the juvenile (immature) meristems, whereas the adult vegetative meristems are able to do so but fail to achieve the threshold level of flowering genes transcription required to flower (Blázquez *et al*., 1997), due to the repression of the *CiFT2* expression in the leaf. We also used flowering adult trees ('off' trees) for comparison. The time-course expression was determined from *Citrus* orthologues of *A. thaliana* flowering time genes (*FT*, *FD*, and *SOC1*), flowering identity genes (*LFY* and *AP1*), flower patterning genes (*SEP1, SEP3* and *FUL*), and flowering time inhibitors (*TFL1*, *TEM1* and *FLC*). The aim of this research is to study the mechanism which impedes the transition of the meristems from the vegetative to the reproductive stage in not flowering citrus trees, either juvenile and adult.

MATERIALS AND METHODS

Plant material and tissue collection

In a first experiment, the time-course of flowering genes expression during the floral bud inductive period was studied. The experiment involved 10-year-old 'Moncada' mandarin trees (adult) and 6-month-old 'Cleopatra' mandarin trees (juvenile). 'Moncada' is a parthenocarpic hybrid mandarin ['Oroval' (*Citrus clementina*) x 'Kara' (*C. unshiu* x *C. nobilis*)] that is known for its absolute biennial bearing (Muñoz-

Fambuena *et al*., 2011). Adult trees flower profusely in spring and set a heavy fruit yield ('on' trees), do not flower in the following spring and develop only vegetatively ('off' trees). Five trees in each condition ('on' and 'off' trees) were selected for the experiments. In this research, 'on' trees are called adult not-flowering trees (A-NFL) whereas 'off' trees are called adult flowering trees (A-FL). Trees were grafted onto 'Carrizo' citrange (*Citrus sinensis* x *Poncirus trifoliata*) rootstock, planted 5 m x 5 m apart in a loamy clay soil, with drip irrigation in the IVIA Research Station (Moncada, Spain). 'Cleopatra' mandarin (*Citrus reshni*) is a self-compatible species that flowers profusely in the adult stage and produces seedy fruits. Fifty seeds were germinated indoors at 22ºC, and the 6-month-old potted plants (juvenile trees) (J-NFL) were transferred to the field in the IVIA Research Station under the same conditions as the adult trees.

Leaf and bud samples to study flowering gene expression were collected from September to February. It is important to note that although plants had different ages, all the buds studied in this experiment were 6-7-month-old, i.e. buds produced in spring (April) were sampled in autumn (September). Five samples per date and plant were taken. In the case of juvenile trees, all the buds (the apical and the axillary buds) and leaves of the trees were sampled. Samples were immediately ground and stored at -80ºC until analysed. Bud sprouting and flowering were evaluated in the spring.

A second experiment to study the transition of the meristem from juvenile to adult stage was conducted using seedlings, juvenile and adult trees of 'Carrizo' citrange, which show a 7-year long juvenile phase, on average (Spiegel-Roy and Goldschmidt, 1996). Six-month-old seedlings and 1-, 3-, 5-, 7-, and 10-year-old trees were used in the experiment. Six biological replicates per tree were used. A sufficient quantity of buds were sampled in February, just before bud differentiation, for the gene expression

analysis. In order to study the influence of age on the sensitivity to chilling as an inducer of flowering, six trees of each age were forced to flower by placing them, on November 10, in a culture chamber with controlled temperatures (15ºC/5ºC day/night), photoperiod (8h/16h day/night), and relative humidity (90%). At 0, 15, 30 and 45 d of cold treatment, leaves from each tree were sampled for gene expression analysis. In all cases, samples were immediately ground and stored at - 80ºC until analysed. Bud sprouting and flowering were recorded in the spring.

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 OD260/OD280 ratio and gel electrophoresis. RNA concentration was determined by fluorometric assays with the RiboGreen dye (Molecular Probes, Engene, Oregon, 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 units of MultiScribe Reverse Transcriptase (Applied Biosystems, Carlsbad, CA, USA), 1 unit of RNase Inhibitor (Applied Biosystems, Carlsbad, CA, USA), 2 µl LC FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostic, 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 conducted at 48 ºC for 30 min, 95 ºC for 10 min followed by 45 cycles at 95 ºC for 2 s, 58 ºC for 8 s and 72 ºC for 8 s.

Fluorescent intensity data were acquired during the 72 ºC-extension step and transformed into relative mRNA values using a 10-fold dilution series of RNA sample as standard curve. Relative mRNA levels were then normalized to total mRNA amounts and, in each case, an expression value of 1 was arbitrarily assigned to the sample which showed the lowest Ct value. *ACTIN* was used as the reference gene according to Mafra *et al*. (2012). Specificity of the amplification reactions was assessed by post-amplification dissociation curves and by sequencing the reaction product. Putative genes were identified through a homology search with related genes from an EST database of entry available in Phytozome V12.1 (https://phytozome.jgi.doe.gov), the species selected being *C. clementina*. Synthetic oligonucleotides were designed in order to amplify the gene of the selected clones and, as stated before, sequenced for confirmation. Details about the forward and reverse primers are given in supplementary **Table S1**. All the genes analysed were described in other studies (see references in Table S1) except the putative candidates for *FLC*, *FD* and *TEM1*. In the *C. clementina* genome, the sequence Ciclev10033420m (*FLC-like*) is quite similar to the *FLC* and *PEP1* genes from *A. thaliana* and *Arabis alpina*, respectively, and was recently described by Hou et al., 2014. The sequence Ciclev10031846m (*TEM1-like*) shows a high similarity to the *TEM1* gene from *A. thaliana* and, also, *Olea europea* (olive tree), which was described by Sgamma et al. 2014. We also studied the expression of the putative homolog to the *FD* gene in the *C. clementina* genome. The sequence Ciclev10003845m (*FD-like*) encoded for a b-Zip transcription factor and showed similarity to the *FD* and *VEG2* (*FD* ortholog) genes from *A. thaliana* and *Pisum sativum*, respectively (Table 1).

Statistical analyses

Gene expression was statistically tested by analyses of variance (ANOVA), using the Student-Newman-Keuls test for means separation. StatGraphics Plus software for Windows, version 5.1 (Statistical Graphics, Englewood Cliffs, NJ, USA) was used.

RESULTS

Tree flowering behaviour

In *Citrus*, adult trees with heavy fruit load in the previous year showed extremely low flowering in the following spring (0.2 flowers 100 nodes^{-1}, on average) (adult-not-flowering trees, A-NFL), whereas those without fruits flowered profusely (149 flowers 100 nodes^{-1}, on average) (adult flowering trees, A-FL). Juvenile trees (J-NFL) did not produce any flower.

Time-course of flowering gene expression during the floral bud inductive period

The time-course expression of *CiFT2* in the leaves of the A-FL trees increased continuously in Spain's mild winter climate from September to February (Figure 1A). At the time of the floral bud inductive period (mid-November), coinciding with the decrease in the average temperature (up to 12 ºC), the *CiFT2* relative expression was significantly upregulated (x1700) and increased until the end of February (x5000). However, in the leaves of the J-NFL tree, *CiFT2* expression was significantly lower in November (x400) compared to the A-FL tree (Figures 1A and 1B). Finally, in the leaves of the A-NFL tree, *CiFT2* expression was completely hampered during the floral bud

inductive period (November), increasing (x250) in January only after fruit harvest (Figure 1B).

We also studied the expression of putative homologs to the *FLC* and *TEM1* genes, which are *FT* inhibitors. The expression of the *FLC-like* gene from *C. clementina* was significantly higher in the leaves of A-NFL (16.5) trees compared to the A-FL (3.8) and J-NFL (4.2) trees during the floral bud inductive period (November) (Figure 1C). The *TEM1-like* gene showed significantly higher expression from September to November in the leaves of NFL trees (juvenile and adult trees) compared to A-FL trees (Figure 1D).

Transcription in the buds of flowering time, identity or patterning genes was strongly affected by the age of the plant. Thus, in most of the studied genes, no transcription was detected in the meristems of juvenile trees (Fig. 2A, B, C, D, H, I). *AtSOC1* orthologues, *CsSL1* and *CsSL2,* upregulated in the meristems of adult trees, whereas they showed no transcription in those of the juvenile trees. *CsSL1* was significantly upregulated (x12) in adult trees from September to February, irrespective of the presence of fruit, and *CsSL2* peaked in mid-November, much higher for A-NLF trees (x5) (Fig. 2A, B). *FD-like* expression was also strongly affected by the age of the plant, since no transcription was detected in the meristems of juvenile trees. However, it slightly downregulated from September to mid-November and upregulated from mid-November to February in both A-FL and A-NFL trees (Fig. 2C).

The relative expression in the bud of the meristem-identity citrus genes *CuFUL*, *CsAP1* and *CsLFY* differed significantly between flowering and not-flowering trees. As expected, *CsAP1* and *CsLFY* genes were upregulated from September to February in the A-FL trees, achieving a significantly higher transcription level in February. The expression of these genes in the A-NFL and J-NFL trees did not differ significantly,

both achieved a significantly lower expression in February compared to the A-FL trees (Fig. 2E, F). A behaviour similar to *CsLFY* was observed for *CuFUL*, but surprisingly, it did not differ significantly between A-FL and A-NFL trees. Transcription of this gene in the J-NFLwas negligible (Figure 2D).

The expression of the flowering inhibitor *CsTFL1* gene in November was significantly higher in the meristem of not-flowering trees than in that of A-FL trees (Fig. 2G). *CsTFL1* presented the highest expression in juvenile buds in September (60% higher than adult trees), and diminished progressively, becoming not significantly different to that of adult trees, A-FL and A-NFL trees, in February.

Finally, the expression of the flower-patterning genes *CiSEP1* and *CiSEP3* was also strongly influenced by the age of the tree and flowering ability. Both genes significantly decreased their expression from September to mid-November, followed by a significant upregulation (x27 and x130, respectively) from November to February in the A-FL meristem. Surprisingly, *CiSEP1* was also upregulated (x15) in the A-NFL meristem. By contrast, *CiSEP1* and *CiSEP3* transcription was hampered in the juvenile meristems (Fig. 2H, I).

Tree age and flowering gene relative expression relationships

To determine when a juvenile tree transcribes flowering time, identity, and patterning genes due to chilling, the relative expression of *CiFT2* was studied in leaves during the floral bud inductive period (November), and that of *FD-like*, *CsSL1, CuFUL, CsLFY, CsAP1*, *CiSEP1* and *CsTFL1* in buds just before the flower bud differentiation stage (February), in trees of different ages.

In our experiment, the *CiFT2* gene expression increased over time, the magnitude of the expression depending on the tree age. Thus, for ½ year-old seedlings the expression was upregulated five times at 30d (480 chilling hours) of cold treatment (15ºC/5ºC, 8h day/ 16h night), and eight times at 45d (720 chilling hours), whereas for 1-year-old and 3-year-old trees it upregulated higher (x16 and x36, respectively), the latter being similar to those for 10-year-old trees (Fig. 3). Therefore, the *CiFT2* gene expression in leaves increased as the age of the J-NFL tree did up to the 3-year-old, making the expression similar to that of 10-year-old A-FL trees.

In the buds, a direct significant relationship between tree age and floral bud inductive gene expression was also found. A significant regression was fitted for *FD-like*, *CsLFY, CsAP1* and *CiSEP1* genes, (r^2=0.78, r^2=0.66, r^2=0.77 and r^2=0.81, respectively), and for *CsSL1* and *CuFUL* genes (r^2=0.86 and r^2=0.76, respectively) (Figure 4). *CsTFL1* expression in the buds showed a negative relationship with tree age.

Nevertheless, considering the population of J-NFL trees only, *FD-like* and *CsLFY* gene expression did not show a significant relationship with tree age (Figures 3A and D), since their expression did not show significant differences between ½ to 7-year-old trees. However, a significant and direct relationship with J-NFL tree age showed *CsAP1*, *CsSL1*, *CuFUL*, *CiSEP1 and FLC-like* genes (r^2=0.61, r^2=0.80, r^2=0.66, r^2=0.98, r^2=0.78) (Figures 3B, C, E, G), and an inverse relationship for the *CsTFL1* gene (R^2=0.91, Figure 3I). *TEM1-like* did not correlated tree age.

DISCUSSION

Flower formation is conferred by four networking processes (Weigel, 1995; Blázquez *et al.,* 2006): (1) the location of newly emerging primordia, (2) the correct timing for the

formation of flowers, (3) the floral identity of the primordia, and (4) the outgrowth of the flower with the correct patterning. Our results show that 6-month-old meristems from juvenile citrus trees (6-month-old seedlings) were unable to transcribe flowering time and flower patterning genes (*SL1*, *SL2*, *FD-like*, *CuFUL*, *CiSEP1* and *CiSEP3*), i.e. to fulfil the networking processes 2 and 4, whereas the 6-month-old meristems from A-NFL trees were able to do it, although they failed to achieve the threshold level of transcription required to flower. This is similar to *Arabidopsis*, which during the juvenile vegetative phase the expression of flowering time genes in the meristems, under LD inductive conditions, is very short (6-7 d), and 14-15 d after germination the plant achieves the level of transcription required to flower (Blázquez *et al*., 1997; Valentim *et al*., 2015). We also found that the juvenile meristem does not achieve the four networking processes equally during maturation.

Long-term meristem maturation

In citrus, *FD-like* and *CsLFY* genes of ½ to 7-year-old trees did not show any significant variation in their expression just before floral bud differentiation, but that for 10-year-old A-FL trees upregulated eight times and twenty times, respectively (Figure 4). This result is similar to that obtained by Valentim *et al*. (2015) for *Arabidopsis* wild-type plants that exhibited an exponential trend for these two gene expressions during the juvenile to adult transition, taking into account the difference in the time scale of these two species, days for *Arabidopsis*, years for citrus. However, the expression of other genes from the flowering program, i.e. *CsSL1*, *CuFUL*, *CsAP1*, *CiSEP1*, and *CsTFL1*, was progressively modified with the age of the tree. These results support the hypothesis that the four genetic programs that confer flower formation (spatial, time,

identity and patterning) might not necessarily occur sequentially (Blázquez *et al*., 2006), and thus demonstrates that the meristems in the juvenile tree progressively achieve a *mature vegetative stage*, with age-dependent differences in the flowering network genes expression. The specific flowering time is mainly uncoupled by a set of key genes (at least *FD-like* and *LFY* in our experiment), which play a fundamental role in the final decision to flower, while others maintain the vegetative stage. In monocarpic plants, the coordinated arrest of all meristems, a process called global proliferative arrest (GPA), which is phenotypically similar to the mature vegetative stage of our juvenile plants, is controlled by an age-dependent upregulation of the FUL transcription factor (Balanzá et al., 2018). In our experiments, an age-dependent upregulation of *CuFUL* was also observed during the mature vegetative stage in juvenile trees (Figure 4C).

Meristem and leaf role during the floral bud inductive period

The level of *AP1* and *LFY* transcription determines flower initiation in *Arabidopsis* (Blázquez *et al*., 1997). In wild-type *Arabidopsis* plants, this threshold is achieved 2-3 days after the increase in flowering time gene expression (Valentim *et al*., 2015). Unexpectedly, our 6-month-old J-NFL trees were able to transcribe *LFY* and *AP1* genes in the meristem, from November to February, at least up to the same level (6 - 7 times increase) as the 6-month-old meristems of the A-NFL trees. Neither the juvenile nor the adult trees flowered, and the only difference between them was the presence of fruits, whereas A-FL did so. Therefore, it seems that transcription of *LFY* and *AP1* genes is not only dependent on juvenility but also on the presence of fruit, as previously shown by (Muñoz-Fambuena *et al*., 2012).

Results suggest that the flowering-time program prevails over the meristem identity program. In citrus, *LFY* and *AP1* overexpression in transgenic 'Carrizo' citrange seedlings reduced flowering time to 12-20 months, but they needed the low-winter temperature signal to upregulate *CiFT2* expression and induce flowering (Peña *et al*., 2001). Moreover, ectopic expression of 35S:*CiFT2* in citrus seedlings produced (i) extremely reduced flowering time (up to 16 weeks), and (ii) continuous flowering regardless of exogenous inductive conditions (Endo *et al*., 2005), reinforcing the role of the flowering time-program in the control of juvenility in *Citrus*. In our experiments, the flowering-time program in the meristem significantly differed between trees. *FD-like*, *CsSL1* and *CuFUL* showed a similar behaviour: (i) upregulation in the adult tree regardless of the flowering phenotype, and no transcription in the J-NFL tree (see Figures 2A, C and D); and (ii) a progressive upregulation of the *SL1* and *CuFUL* genes during the juvenile to adult meristem transition (Figures 4B and C), which is in accordance with their implication in the age flowering pathway of *A. thaliana* (Wang *et al*., 2009), and *Citrus sp.* (Castillo *et al*., 2013). This latter result was not confirmed for the *FD-like* gene, at least in plants between ½ to 7 years-old, suggesting a key role for this gene for the vegetative-to-flowering meristem transition, as recently reported in pea (*P. sativum*) (Sussmilch *et al*., 2015). Moreover, in the adult stage, trees were able to express *FD-like* genes in the bud regardless of *CiFT2* transcription in the leaf (Figure 1), as previously found by Muñoz-Fambuena *et al*. (2012). The phylogenetic analysis showed that the *C. clementina FD-like* was similar to the reported FD proteins (data not shown). The amino acid sequence alignment contains a bZIP domain, which is conserved in other FD proteins (Sussmilch *et al*., 2015). Therefore, it likely forms a florigen activation complex with *FT* genes, as reported previously (Sussmilch *et al*., 2015), althought further studies are needed in this regard.

Pilliteri *et al*. (2004) compared *CsTFL1* gene expression in 4-month-old citrus seedlings and A-FL trees during eleven weeks, and found a correlation between *CsTFL1* gene expression and juvenility. Our results regarding 6-month-old trees agree with the latter, but further show that *CsTFL1* is not the cause of the lack of flowering in juvenile trees older than 1-year-old, since it was downregulated in juvenile trees from 1 to 7-years-old (Figure 2).

We also found that the flowering time program was impeded in the leaf of both the juvenile and A-NFL trees, which were not able to transcribe the *CiFT2* gene up to the flowering level of the A-FL trees. Nishikawa *et al*. (2007) reported that 4-month-old citrus seedlings were not able to respond to inductive low temperature (15ºC), compared to the adult flowering trees; however, we found response to 15ºC/5ºC *CiFT2* upregulation in juvenile plants from 1/2 to 3 years old (Figures 1 and 3). On the contrary, *CiFT2* expression was nil up to fruit harvest in A-NFL trees (Figure 1), as previously shown by Muñoz-Fambuena *et al*. (2011). In *Arabidopsis* and *Antirrhinum* wild type plants, *FT* is progressively transcribed during the adult vegetative phase, before the transition to the adult reproductive phase determined by *AP1* and *LFY* (Sgamma *et al*., 2014; Valentim *et al*., 2015). Our results suggest that the leaf on the 6-month-old tree might be in transit to the adult vegetative stage, and that the reproductive stage is finally accomplished in the meristem. Supporting this hypothesis is the fact that early overexpression of *CiFT2* in transgenic citrus lead to the conversion of vegetative shoots into leafy inflorescences rather than the transition from the juvenile to adult phases (Endo *et al*., 2005). Taken together, our results suggest that the inability of the meristem to differentiate may be the main constraint of flowering in the juvenile tree, whereas it is the fruit blocking the *CiFT2* expression in the leaf the main constraint for flowering in the adult tree. Further support for this hypothesis is the fact that *FLC-like*

was scarcely expressed in leaves from young trees compared to adult citrus trees, a result also reported by Castillo *et al*. (2013), and its expression was the highest in adult trees with fruits. The flowering inhibitor FLC is a transcription factor that acts in the vascular tissue to bind directly to the *FT* gene and to repress its transcription (Michaels and Amasino, 1999). However, a clear relationship between these two genes has yet to be established in fruit trees (Andrés and Coupland, 2012).

Conclusion

Accordingly, in *Citrus sp.*, axillary meristems of juvenile trees are unable to transcribe flowering time and flower patterning genes (*FD-like, CsSL1*, *CuFUL*, *SEP1* and *SEP3*). Meristems progressively achieve the flowering time program, and when they flower for the first time the derived meristems are always able to transcribe these genes, except when the adult tree produces a heavy crop load. In these cases, the axillary meristem is able to transcribe the flowering time-program genes, but fails to achieve the particular level of *CiFT2* transcription required to flower (Figure S1).

We propose that genetic inhibition of flowering time in juvenile tree is determined in the meristems and this is due to its inmaturity, whereas in an adult tree it is determined in the leaf, where repression of *CiFT2* gene expression occurs.

FUNDING

This work was supported by a grant from the 'Instituto Nacional Investigaciones Agrarias (INIA)', Spain [RTA2013-00024-C02-02].

ACKNOWLEDGEMENTS

We thank Cristina Ferrandiz (IBMCP-UPV, Spain) and Fernando Andrés (UMR AGAP, France) for useful comments on the manuscript. We thank D. Westall for her help in editing the manuscript.

LITERATURE CITED

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** (5737): 1052-1056.

Albani MC, Coupland G. 2010. Comparative analysis of flowering in annual and perennial plants. *Current Topics in Developmental Biology* **91**: 323-348.

Andrés F, Coupland G. 2012. The genetic basis of flowering responses to seasonal cues. *Nature Reviews Genetics **13***: 627-639.

Balanzá V, Martínez-Fernández I, Sato S, et al. **2018**. Genetic control of meristem arrest and life span in *Arabidopsis* by a FRUITFULL-APETALA2 pathway. *Nature Communications*, 9: 565.

Bäurle I, Dean C. 2006. The timing of developmental transitions in plants. *Cell* **125**: 655-664.

Betancourt M, Sistachs V, Martinez-Fuentes A, Mesejo C, Reig C, Agusti, M. 2014. Influence of harvest date on fruit yield and return bloom in 'Marsh'grapefruit trees (*Citrus paradisi* Macf.) grown under a tropical climate. *The Journal of Horticultural Science and Biotechnology* **89**: 435-440.

Blázquez MA, Soowal LN, Lee I, Weigel, D. 1997. LEAFY expression and flower initiation in *Arabidopsis*. *Development* **124**: 3835-3844.

Blazquez MA, Ferrandiz C, Madueno F, Parcy F. 2006. How floral meristems are built. *Plant MolecularBbiology*, **60**: 855-870.

Bluemel M, Dally N, Jung C. 2015. Flowering time regulation in crops—what did we learn from *Arabidopsis*?. *Current Opinion in Biotechnology* **32**: 121-129.

Castillo MC, Forment J, Gadea J, et al. **2013**. Identification of transcription factors potentially involved in the juvenile to adult phase transition in Citrus. *Annals of Botany*, **112**: 1371-1381.

Chica EJ, Albrigo LG. 2013. Expression of flower promoting genes in sweet orange during floral inductive water deficits. *Journal of the American Society for Horticultural Science*, **138**: 88-94.

Davies FS, Albrigo LG. 1994. *Citrus*. CABI International. Wallingford, U.K.

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.

Haberman A, Ackerman M, Crane O, Kelner JJ, Costes E, Samach A. 2016. Different flowering response to various fruit loads in apple cultivars correlates with degree of transcript reaccumulation of a TFL1-encoding gene. *The Plant Journal* **87**: 161-173.

Hanano S, Goto K. 2011. *Arabidopsis* TERMINAL FLOWER1 is involved in the regulation of flowering time and inflorescence development through transcriptional repression. *The Plant Cell* **23**: 3172-3184.

Mafra V, Kubo KS, Alves-Ferreira M, et al. 2012. Reference genes for accurate transcript normalization in citrus genotypes under different experimental conditions. *PloS One* **7**: e31263.

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.

Muñoz-Fambuena N, Mesejo C, Carmen González-Mas M, 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, Primo-Millo E, Agustí M, Iglesias DJ. 2012. Fruit load modulates flowering-related gene expression in buds of alternate-bearing 'Moncada'mandarin. *Annals of Botany* **110**: 1109-1118.

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.

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

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

Seo E, Le, H, Jeon J, et al **2009**. Crosstalk between cold response and flowering in *Arabidopsis* is mediated through the flowering-time gene *SOC1* and its upstream negative regulator FLC. *The Plant Cell* **21**: 3185-3197.

Sgamma T, Jackson A, Muleo R, Thomas B, Massiah A. 2014. TEMPRANILLO is a regulator of juvenility in plants. *Scientific Reports*, 4: 3704 DOI: 10.1038/srep03704

Shalom L, Samuels S, Zur N, et al 2012. Alternate bearing in citrus: changes in the expression of flowering control genes and in global gene expression in on-versus off-crop trees. *PLoS One* 7: e46930.

Shalom L, Samuels S, Zur N, et al **2014**. Fruit load induces changes in global gene expression and in abscisic acid (ABA) and indole acetic acid (IAA) homeostasis in citrus buds. *Journal of Experimental Botany* **65**: 3029-3044.

Sohn EJ, Rojas-Pierce M, Pan S, et al **2007**. The shoot meristem identity gene TFL1 is involved in flower development and trafficking to the protein storage vacuole. *Proceedings of the National Academy of Sciences*, **104**: 18801-18806.

Spiegel-Roy P, Goldschmidt EE. 1996. *The biology of citrus*. Cambridge University Press.

Sussmilch FC, Berbel A, Hecht V, et al **2015**. Pea VEGETATIVE2 is an FD homolog that is essential for flowering and compound inflorescence development. *The Plant Cell*, **27**: 1046-1060.

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

Valentim FL, van Mourik S, Posé D, et al **2015**. A quantitative and dynamic model of the *Arabidopsis* flowering time gene regulatory network. *Plos One*, **10**: e0116973.

Wang JW, Czech B, Weigel D. 2009. miR156-regulated SPL transcription factors define an endogenous flowering pathway in *Arabidopsis thaliana*. *Cell*, **138**: 738-749.

Weigel, D. 1995. The genetics of flower development: from floral induction to ovule morphogenesis. *Annual Review of Genetics* 29: 19-39.

CAPTIONS TO FIGURES

FIG. 1**.** Time-course of *CiFT2* (A, B) *FLC-like* (C) and *TEM1-like* (D) genes expression in the leaves of juvenile (NFL-juvenile) and adult mandarin trees with heavy fruit load (*on* trees, NFL-Adult) or whithout fruit (*off* trees, FL-Adult). Data are mean of three independent replicates. In all cases, bars of SE are smaller than symbol size. Different letters for a given month indicate significant differences ($P<0.05$).

FIG. 2**.** Time-course of *CsSL1 (A), CsSL2 (B), FD-like (C), CiFUL (D), CsLFY (E), CsAP1 (F), CsTFL1 (G), CiSEP1 (H) and CiSEP3 (I)* genes expression in the buds of juvenile (NFL-juvenile) and adult trees with heavy fruit load (*on* trees, NFL-Adult) or whithout fruit (*off* trees, FL-Adult). Data are mean of three independent replicates. In all cases, bars of SE are smaller than symbol size. Different letters for a given month indicate significant differences ($P<0.05$).

FIG. 3**.** Time-course of *CiFT2* gene expression in leaves of 0.5, 1 and 3-years-old juvenile (NFL-J) and 10-years-old adult 'Carrizo' citrange trees (FL-A). Data are mean

of three independent replicates. Vertical bars indicate the standard error. Different letters for a given plant age indicate significant differences ($P<0.05$).

FIG. 4. Time-course of *FD-like (A),CsSL1 (B), CiFUL (C), CsLFY (D), CsAP1 (E), CiSEP1 (F, G) and TEM1-like (H), CsTFL1 (I)* and *FLC-like (J)* genes expression in the buds of 0.5, 1, 3, 5, and 7-years-old juvenile (NFL-J) and 10-years-old adult 'Carrizo' citrange trees (FL-A). Data are mean of three independent replicates. Vertical bars indicate the standard error. Different letters indicate significant differences ($P<0.05$).

1

2 **TABLES**

3

4 TABLE 1. Sequence analysis of the putative FD, FLC and TEM homolog genes from *Citrus clementina*.

Annotation	EST code	Homologous locus	BLASTP (E- value)	Qc (%)
FD-like	Ciclev10003845m	FD (*A. lyrata*)	2 e^{-20}	92
		VEG2 (*P.sativum*)	1 e^{-20}	92
FLC-like	Ciclev10033420m	*FLC-like* (*C. sinensis*)	4 e^{-93}	99
		K-box region MADS-box transcription factor family (*A. thaliana*)	2 e^{-7}	83
TEM1-like	Ciclev10031846m	RAV1 (*A. thaliana*)	2 e-150	98
		TEM1 (*O. europea*)	0.0	98

5

6

7