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Fruit-dependent epigenetic regulation of flowering in *Citrus*

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Summary

- In many perennial plants, seasonal flowering is primarily controlled by environmental conditions, but in certain polycarpic plants, environmental signals are locally gated by the presence of developing fruits initiated in the previous season through an unknown mechanism.
- Polycarpy is defined as the ability of plants to undergo several rounds of reproduction during their life time, alternating vegetative and reproductive meristems in the same individual.
- To understand how fruits regulate flowering in polycarpic plants, we have focused on alternate bearing in *Citrus* trees that had been experimentally established as fully flowering or non flowering.
- We have found that the presence of the fruit causes epigenetic changes correlating with the induction of the *CcMADS19* floral repressor, which prevents the activation of the floral promoter *CiFT2* even in the presence of the floral inductive signals. In contrast, newly emerging shoots display an opposite epigenetic scenario associated with *CcMADS19* repression, thereby allowing the activation of *CiFT2* the following cold season.

Keywords: Alternate bearing, *CcMADS19*, Citrus, flowering, *FT*, chromatin remodeling

Introduction

According to their reproductive behaviour, plants and animals can be divided in two groups. Semelparity describes those organisms that divide only once in their life time, while iteroparity defines the ability to reproduce multiple times (Cole, 1954; Charnov & Schaffer, 1973). In the green lineage, semelparity is frequent among herbaceous plants which flower at a specific time of the year and then senesce (*i.e.* monocarpic plants), while iteroparity is habitual in some herbaceous species and most woody angiosperms which produce flowers once a year during multiple seasons (*i.e.* polycarpic plants). The key characteristic of polycarpic plants is that they alternate vegetative and reproductive meristems in the same individual, and the molecular mechanism by which these two fates are controlled is still intriguing (Bratzel & Turck, 2015).

In annual plants, photoperiod (Suarez-Lopez *et al.*, 2001), vernalization (Sheldon *et al.*, 2000), and ambient temperature (Blázquez *et al.*, 2003) affect the expression of the floral pathway integrator *FLOWERING LOCUS T (FT)*, determining the correct time of flowering. Summer annual plants flower and develop rapidly when grown under long days, whereas winter annuals can grow for months under long days without flowering (Andres & Coupland, 2012). The latter avoid flowering in unfavorable conditions by blocking the response to inductive signals by the MADS domain transcription factor *FLOWERING LOCUS C (FLC)* and its homologs that directly repress genes related to floral transition (Sheldon *et al.*, 2000). After a shift to cold temperatures, chromatin modifications stably repress *FLC* transcription, and this repression persists after vernalization (Finnegan & Dennis, 2007).

The best studied case of polycarpic development is that of *Arabis alpina*, a perennial herbaceous plant in which the expression of the *FLC* ortholog *PERPETUAL FLOWERING1 (pep1)* is transiently repressed by cold temperature to allow flowering in the subsequent season, but then undergoes upregulation by warm temperature to limit flowering only to the spring season (Wang *et al.*, 2009; Bratzel & Turck, 2015). However, it has been shown that the response to vernalization is efficient only after certain age of the plant, and work with *A. alpina* and the biennial-to-perennial plant *Cardamine flexuosa* indicates that this gating mechanism depends on two age-regulated microRNAs (Bergonzi *et al.*, 2013; Zhou *et al.*, 2013).

A very different case of polycarpic behaviour is that of fruit trees, like citrus, avocado, mango, pecan, olive or apple, in which the inductive effect of environmental signals is locally repressed by the presence of developing fruits initiated in the previous season (Martinez-Fuentes *et al.*, 2010), probably as a strategy to optimize resource allocation throughout the plant (Martinez-Alcantara *et al.*, 2015). In *Citrus*, for instance, cold temperature during fall induces flowering in the Mediterranean climates (Liebig & Chapman, 1963) whereas in tropical areas flowering is induced by water stress (Cassin *et al.*, 1969). Both stimuli have been associated with a seasonal increase of the expression of *Citrus* orthologue of *FT (CiFT2)* (Nishikawa *et al.*, 2007; Chica & Albrigo, 2013). Interestingly, fruit remaining on the tree during the floral bud inductive period correlates with reduced levels of the *CiFT2* gene expression (Munoz-Fambuena *et al.*, 2011). Although fruit-dependent inhibition of flowering is a local response, affecting only the newly generated shoots in the vicinity of developing fruits, in some extreme cases, a season with heavy fruit yield (the ON season) is accompanied by no flowering in the whole tree and, consequently, a season with no fruit

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production (the OFF season). This behaviour is agronomically known as “alternate bearing” and it represents potentially large economic losses in Agriculture. This particular polycarpic habit that results from the interplay between endogenous and environmental signals cannot be understood only on the basis of knowledge acquired through the studies with herbaceous plants in which fruits have not been described to alter reproductive behaviour. Therefore, we have approached this issue directly in citrus trees, and here we describe how fruit-dependent epigenetic regulation of a flowering repressor encoded by *CcMADS19* correlates with the ability of *CiFT2* expression to respond to environmental signals in proximal leaves.

Materials and Methods

Plant material and growth conditions

Experiments were carried out using field grown 18-year-old trees of ‘Moncada’ mandarin [Clementina Oroval (*Citrus clementina* Hort. ex Tan.) x ‘Kara’ mandarin (*C. unshiu* Marc. x *C. nobilis* Lou.)] and 12-year-old ‘Afourer’ tangor (*C. reticulata* x *C. sinensis*), grafted onto Carrizo citrange [*C. sinensis* (L.) Osbeck x *Poncirus trifoliata* (L.) Raf.] rootstock, and exhibiting a marked alternate bearing. Trees were planted 5 m x 5 m apart, drip irrigated, fertilized, and grown according to usual techniques. Experimental field was located in the IVIA Research Station (Moncada, Spain). *Arabidopsis thaliana* seeds (Col-0) were surface sterilized and grown in a growth room under 16 h light (150-200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and 8 h dark cycle at 22°C. All molecular analyses were performed in the same year, unless specified.

Tree phenotyping

The effect of fruit load on flowering was studied on 6 ON (fully loaded) and 6 OFF (without fruit) trees randomly selected according their uniformity in size and vigor. Flowering intensity was evaluated in spring by randomly selecting four branches per tree of three ages (late spring, summer and autumn sprouts), in all directions, and with some 300 nodes per branch. The number of sprouted nodes, sprouts, and the flowers per sprout were counted, giving the results as the number of flowers per 100 nodes to compensate for the differences in size of the selected branches. In summer and fall, number of vegetative shoots was counted from the same branches, referring the results also per 100 nodes. Total yield per tree was determined by weighing all fruits at harvest (February). Defruiting experiments were performed on another set of 6 ON-trees. All fruits of the trees were removed at the onset of stage II of fruit development (July). From mid-May to the end of February, 10 leaves per tree

from the spring flush were collected at 11 a.m. for RNA extractions. In mid-January, 30 buds per each kind of tree were also sampled at 11 a.m. for RNA extraction. Samples were immediately ground and stored at -80°C until analyses. The effect of the methyltransferase inhibitor 5-azacytidine (5-aza, 350 µM) on flowering genes expression was studied on 3 ON-trees treated three times (September, October and November). A nonionic surfactant Tween[®] 20 (polyethylene glycol sorbitan monolaurate, Sigma-Aldrich Química, Madrid, Spain) at a concentration of 0.02% was added to the solution. Young leaves (2-month-old) were sampled at 0, 24 and 48 h after the last treatment. Untreated trees were used as control for comparison.

Sequence analysis

Amino acid sequences of the genes studied were obtained from the Phytozome v10.3 database (www.phytozome.net). Multiple sequence alignment and phylogram analysis were carried out with the Clustal Omega tool at NCBI (www.ebi.ac.uk/Tools/msa/clustalo/).

Gene expression analysis

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 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, Eugene, Oregon, USA) according to manufacturer's instructions. cDNA was obtained from 1 µg total RNA using the QuantiTect[®] Reverse Transcription Kit (Qiagen, USA) in a total volume of 20 µl. Quantitative real-time PCR was carried out on a Rotor Gene Q 5-Plex (Qiagen, USA) using the QuantiTect[®] SYBR[®] Green PCR Kit (Qiagen, USA). The reaction mix and conditions followed the manufacturer's instructions with certain modifications. The PCR mix contained 2.5 µl of a 4-fold cDNA dilution, 12.5 µl of QuantiTect[®] SYBR Green PCR Master Mix (Qiagen, USA), 1.5 µl of 0.3 µM primer F, and 1.5 µl of 0.3 µM primer R, the final volume being 25 µl. The cycling protocol for the amplification consisted of 15 min at 95°C for pre-incubation, then 40 cycles of 15 s at 94°C for denaturation, 30 s at 60°C for annealing and 30 s at 72°C for extension. The sequences of the primers used are presented in Supplementary Table 1.

Bisulphite sequencing

Genomic DNA (450-750 ng) was treated with sodium bisulphite using the EpiTect Bisulphite kit (Qiagen) according to the manufacturer's instructions. The reaction was then purified once more using the PCR purification kit (Qiagen, USA). The bisulphite treated DNA was amplified using Hot start *Platinum® Taq* DNA Polymerase (Invitrogen). Primer sequences are presented in Supplementary Table 1. The thermal cycling program was set at 95°C for 1 min followed by 40 cycles of 95°C for 30 s, annealing 50° for 30 s, and extension at 65-72°C for 30 s, ending with a 3 min extension at 65-72°C. DNA fragments were cloned into pGEM-T (Promega) before sequencing at least 10 different clones.

CcMADS19 gene cloning and plant transformation

The full-length coding sequence of *CcMADS19* was amplified by PCR using as template a clone from IVIA1 library (Forment *et al.*, 2005), IC0AAA56AF11, with primers in Supplementary Table 1, cloned in pCR8/GW/TOPO® TA vector (Invitrogen), and then mobilized into pEarlyGate201 (Earley *et al.*, 2006) by LR reaction with Gateway® LR Clonase® II (Invitrogen). The full genomic *CcMADS19* was deposited in the GeneBank with reference number MN119275. Before plant transformation the construct was introduced into *Agrobacterium tumefaciens* C58 cells. Arabidopsis plant transformation was carried out by the “floral dip” method (Clough & Bent, 1998).

Citrus agroinfiltration

Transient expression experiments in citrus leaves were performed as previously described, with sequential infection by *Pseudomonas* and *Agrobacterium* (Jia & Wang, 2014). Briefly, leaves from OFF trees were inoculated with either tap water or a culture of *Pseudomonas syringae* (10^1 , 10^2 , 10^4 and 10^8 CFU/ml) resuspended in sterile tap water (5×10^8 CFU/ml). Sixteen hours later, the same inoculated leaf areas were subjected to agroinfiltration as described previously. Recombinant *Agrobacterium tumefaciens* cells were cultured in 3-ml Luria broth (LB) medium with appropriate antibiotics at 28°C. A new 100-ml fresh LB medium culture was inoculated with 100 µl of the overnight culture, including 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.6, and 40 mM acetosyringone (AS), as well as the appropriate antibiotics. Upon reaching OD600 = 0.8, the inoculum was harvested and resuspended in MMA solution (10 mM MgCl₂, 10 mM MES, pH 5.6 and 200 mM AS) to a final OD600 of 1.0. The suspension was left at room temperature for 2 h and infiltrated into

the same area previously inoculated with *P. syringae*. Citrus leaves agroinfiltrated with *Agrobacterium* in the absence of *P. syringae* inoculation were used as control. The presence of the agroinfiltrated protein was confirmed by western blotting.

Chromatin immunoprecipitation

ChIP was performed as previously described (Lee *et al.*, 2007) with the following modifications. The crude nuclear pellet was resuspended in nuclear lysis buffer and sonicated in a Covaris M220 focused-ultrasonicator for 8 min at 6°C with a 5% duty factor. The soluble chromatin solution was incubated with 1 µg of anti-H3K27me3 (Millipore 07-449) and anti-H3K4me3 (Millipore 07-473) for 4 hours, and chromatin-antibodies complexes were captured with protein A/G magnetic beads (Thermo Scientific). De-crosslinking reaction was performed with Chelex slurry (Biorad) as described (Nelson *et al.*, 2006).

For the identification of the H3K27me3 and H3K4me3 regulated regions, we first divided the *CcMADS19* promoter (5000 bp) and genomic region (13800 bp) in bins of 1000 bp, and designed primers to amplify ca. 180 bp within each bin. Nineteen pairs of primers were screened in total by qPCR against the input. We then performed a comparative analysis between induced and non induced samples.

Results and Discussion

CcMADS19 gene expression correlates with fruit mediated flowering inhibition.

Citrus trees of the 'Moncada' mandarin cultivar maintain marked alternate bearing (Munoz-Fambuena *et al.*, 2011). The twelve particular individuals, in two groups, used in our study produced an average of 143 and 0.7 flowers per 100 nodes in the first year, i.e. they were in the ON and OFF state, respectively (Fig. 1a). Right after flowering, the ON trees produced an average yield of 87 kg and the OFF ones hardly produced 10 kg (Fig. 1b). Both groups of trees maintained alternate bearing behaviour during the 4 years of the experiment. Reciprocally, trees in the ON state produced only 53 vegetative shoots m⁻², while OFF trees reached over 160 vegetative shoots m⁻² during the spring, summer and fall flushes (Fig. 1c, d).

Although the orthologs of several genes involved in the promotion of flowering in *Arabidopsis* and other plants have been described in *Citrus* trees (Nishikawa *et al.*, 2007; Shalom *et al.*, 2012), no floral repressors equivalent to *FLC* have been described that could account for the fruit-mediated inhibition of flowering in woody species. Examination of MADS-box phylogenetic trees indicates that the *FLC* clade is ancestral to angiosperms (Ruelens *et al.*, 2013), although members of this group have been lost multiple times (Gramzow & Theissen, 2015). However, *FLC* orthologs appear indistinctly in some species (Supplementary Fig. 1a,b), for instance *Beta vulgaris*, where it has been proposed to be functional in flowering time control (Reeves *et al.*, 2007), and in the genome of fruit trees like *Prunus persica* (Wells *et al.*, 2015) and also *Citrus sinensis* and *Citrus clementina* (Hou *et al.*, 2014). Given that *FLC* family members have been implicated not only in flowering regulation, but also in transitions between growth and dormancy states (Deng *et al.*, 2011; Berry & Dean, 2015), we investigated if the *FLC* ortholog encoding CcMADS19 (Hou *et al.*, 2014) would participate in the fruit-mediated regulation of flowering and alternate bearing.

Temporal analysis of gene expression showed, as previously reported, that the expression of the *CiFT2* gene increased in young leaves formed in spring in OFF trees in response to low temperature that promotes flowering (Moss, 1969; Nishikawa *et al.* 2007), but not in ON trees (Fig. 2a, b). Interestingly, this effect inversely correlated with the expression of *CcMADS19*, which was higher in ON than in OFF trees in the moment when the floral transition is established in OFF trees, i.e. in November-December (Fig. 2c). It is noteworthy that *CcMADS19* expression increased further in both ON and OFF trees, coinciding with the return to warm temperatures (January; Fig. 2 a, c), as reported for *PEP1* in *A. alpina* (Wang *et al.*, 2009). This increase did not interfere with flowering in OFF trees because it occurred after flowering had already been established. It has been suggested that these changes in floral suppressor contribute to the perennial life history (Wang *et al.*, 2009).

The autonomous upregulation in ON compared to OFF trees was specific to *CcMADS19*, since the expression in leaves of *CcMADS42* and *TEMPRANILLO-LIKE1* (*CcTEML1*) whose orthologs in *Arabidopsis*, *SHORT VEGETATIVE PHASE* (*SVP*) and *TEMPRANILLO1* (*TEM1*), respectively, also regulate the floral transition (Hartmann *et al.*, 2000; Sgamma *et al.*, 2014), did not vary significantly between ON and OFF trees during a whole one-year period (Supplementary Fig. 2 a,b).

The dynamics of *CcMADS19* expression in young leaves (low from May to October) suggests that low expression is reprogrammed in the dormant bud and in leaves of newly emerging shoots each season, and it is the presence of mature fruits in ON trees in November which promotes *CcMADS19* expression in the mature (8-month-old) leaves. To confirm this hypothesis, we removed the young fruits as soon as they set in July in ON trees. This manipulation yielded a shift in the status of the defruited (DEF) tree, which then behaved as an OFF tree and allowed the formation of flowers during the subsequent inductive period (Fig. 3a). In accordance, leaves of DEF trees showed similar *CiFT2* and *CcMADS19* gene expression of those of OFF trees, significantly higher and lower, respectively, than those of ON trees (Fig. 3b, c). On the other hand, no significant differences were found between ON, OFF and DEF trees for *CcMADS42* and *CcTEML1* genes (Supplementary Fig. 2c).

***CcMADS19* is a floral repressor that downregulates *CiFT2* expression.**

The observations that (i) *CcMADS19* is an ortholog of *FLC* (Hou, *et al.*, 2014), (ii) it displays a temporal expression pattern opposite to that of *CiFT2*, and (iii) its expression level is enhanced by the presence of fruits, suggests that *CcMADS19* may mediate the fruit-dependent regulation of *CiFT2*. To test this hypothesis, we first expressed the *CcMADS19* cDNA from the CaMV35S promoter in wild-type *A. thaliana* Col-0 plants. The homozygous transgenic plants were late flowering (Fig. 4a) and increased significantly the number of rosette leaves (Fig. 4b), demonstrating that *CcMADS19* can act as a floral repressor in a heterologous background, similar to what has been observed for the *B. vulgaris* *FLC* ortholog (Reeves *et al.*, 2007). More importantly, *CcMADS19* repressed the expression of *CiFT2* when it was transiently expressed in the leaves from OFF citrus trees at the time the floral buds should be established (i.e. November) (Fig. 4c). These results indicate that *CcMADS19* acts as a floral repressor acting, directly or not, on *CiFT2* expression.

Fruit-mediated chromatin remodelling at the *CcMADS19* locus regulates floral induction.

In both *A. thaliana* and *A. alpina* *FLC* and *PEP1* are regulated through chromatin modifications (Finnegan & Dennis, 2007; Wang *et al.*, 2009). Molecular memories can be propagated across mitotic cell divisions, but they must be erased to re-establish sensitivity to external signals that induce flowering (Albani & Coupland, 2010; Jones, 2012; Bratzel & Turck, 2015). Thus, we hypothesized that *CcMADS19* gene expression would correlate with

epigenetic marks, i.e. DNA methylation or histone modifications, in a fruit-dependent manner.

DNA methylation is highly correlated with gene silencing (Jones, 2012). We first studied the DNA methylation profile of *CiFT2*, *CcMADS19*, *CcMADS42* and *TEML1*-like genes. Leaves were sampled at the floral inductive period (November), when *CcMADS19* is differentially expressed in ON and OFF trees (see Fig. 2c). While no difference in cytosine methylation pattern was found between ON and OFF trees in the 7, 8 and 20 CG sites of *CiFT2*, *CcMADS42* and *CcTEML1*, respectively (Supplementary Table 2), we did find significant changes in cytosine methylation in the *CcMADS19* gene. Methylation was examined in three regions (Supplementary Fig. 1c): (i) the proximal promoter (-1000 bp), (ii) intron 1, from +8035 bp to +8421 bp, and (iii) intron 1, from +8858 bp to +9198 bp. In the promoter region, CG sites showed no methylation in either ON or OFF trees, and only the position 21 (CHH), out of 25, showed partial methylation (4 out of 10 clones) in OFF trees (Supplementary Table 2). But in the intron region, ON trees consistently showed differential cytosine methylation with respect to OFF trees: overmethylation in positions 27 (CHH), 29 (CHH) and 31 (CG), and undermethylation in position 33 (CG) (Supplementary Table 2). More importantly, DEF trees rendered a methylation pattern more similar to that of OFF trees (Fig. 5a), indicating a causal connection between the presence of fruits and the DNA methylation status at the *CcMADS19* locus. To confirm the relationship between the methylation pattern and the expression level of *CcMADS19*, we examined the effect of 5-azacytidine (5-aza) on *CcMADS19* and *CiFT2* expression. This chemical is a cytosine analog that inhibits DNA methyltransferases and modifies cytosine methylation and gene expression (Tsuboi et al., 2012). In the ON leaves treated with 5-aza, *CcMADS19* expression underwent a two-fold increase for 24 and 48 h with respect to mock-treated trees, which was accompanied by a similar reduction in *CiFT2* expression (Fig. 5b).

In *A. thaliana*, although DNA methylation of the *FLC* locus affects its expression level, the biologically relevant signal that modulates *FLC* expression, vernalization, does not operate through this mechanism (Finnegan et al., 2005). Given that DNA methylation and histone modifications are usually interdependent (Du et al., 2015) and that in *A. thaliana* and *A. alpina* the activated/repressed state of the *FLC* and *PEP1* genes, respectively, correlate with histone modifications (Wang et al., 2009; Yang et al., 2014; Whittaker & Dean, 2017), we also examined histone modifications in the *CcMADS19* locus in buds of ON, OFF and DEF trees at the time of floral induction (November). The promoter and first intron of *CcMADS19* were evaluated by ChIP-qPCR for enrichment of the H3K4me3 mark, and two

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regions from the promoter consistently displayed differential behaviour between ON and OFF trees. In both cases, this activatory mark was enriched in the leaves of ON trees, *i.e.*, those that do not flower because of the presence of fruits (Fig. 6a). This differential enrichment was probably the cause of the previously observed enhanced expression of *CcMADS19* in ON trees (Fig. 2c), given that in DEF trees, in which young fruits were manually detached, the presence of the H3K4me3 mark was reduced, mimicking OFF trees (Fig. 6a), as was *CcMADS19* expression (Fig. 3c). This result was further supported by the observation that the expression of the citrus orthologs of the methyltransferases *TRITHORAX (TRX)1* and *TRX7*, required for the activation of *FLC* expression in Arabidopsis (Pien *et al.*, 2008; Tamada *et al.*, 2009), correlated with the level of the H3K4me3 mark in ON, OFF and DEF trees (Fig. 6 b).

These results suggest that the presence of the fruit provokes the epigenetic activation of *CcMADS19* in the adjacent mature leaves, to locally and temporally repress *CiFT2* upregulation and, thus, reproductive development in the axillary bud for the subsequent flowering period. However, it does not explain the necessary reprogramming of the buds that will eventually flower in the following season. Considering that this switch has been attributed to epigenetic repression of *FLC* and *PEP1* in *A. thaliana* and *A. alpina*, respectively, during seasonal reprogramming (Wang *et al.*, 2009), we examined the presence of the H3K27me3 mark in the buds of ON and OFF trees the following February, just before spring sprouting. As expected, this repressive mark was enriched in the buds of ON trees (Fig. 6c), suggesting that the lack of upregulation of *CcMADS19* in the buds and new leaves (Fig. 6d) would allow the new emerging vegetative shoots (OFF season) to have positive response to floral inductive signals the following flowering period (ON season).

In summary, our results are compatible with a model in which fruit-dependent epigenetic activation of the *CcMADS19* floral repressor would prevent the activation of the floral promoter *CiFT2* even in the presence of the floral inductive low temperatures. But the axillary bud and its newly emerging shoots would then undergo epigenetic reprogramming resulting in the repression of *CcMADS19*, thereby allowing the activation of *CiFT2* the following cold season (Supplementary Fig. 3). This mechanism resembles the seasonal vernalization switch in perennial herbaceous species, like *A. alpina*, or the generational switch occurring during meiosis in annual species, like *A. thaliana*. However, it is important to remark that, in this case, the responsiveness of meristems to floral inductive signals is established in a fruit-dependent manner. While the logic and the core elements of the mechanism have been conserved in evolution, divergence has occurred at the regulatory

signal that governs the process. Interestingly, fruits have also been shown to regulate other aspects of plant biology, like the lifespan of reproductive meristems in annual species, although in that case shoot apical meristem-specific genes are irreversibly shut off (Balanza *et al.*, 2018). Whether equivalent signals regulate both processes still requires further work.

References

- Albani MC, Coupland G. 2010.** Comparative analysis of flowering in annual and perennial plants. *Curr Top Dev Biol* **91**: 323-348.
- Andres F, Coupland G. 2012.** The genetic basis of flowering responses to seasonal cues. *Nat Rev Genet* **13**: 627-639.
- Balanza V, Martinez-Fernandez I, Sato S, Yanofsky MF, Kaufmann K, Angenent GC, Bemer M, Ferrandiz C. 2018.** Genetic control of meristem arrest and life span in *Arabidopsis* by a *FRUITFULL-APETALA2* pathway. *Nat Commun* **9**: 565.
- Bergonzi S, Albani MC, Ver Loren van Themaat E, Nordstrom KJ, Wang R, Schneeberger K, Moerland PD, Coupland G. 2013.** Mechanisms of age-dependent response to winter temperature in perennial flowering of *Arabidopsis alpina*. *Science* **340**: 1094-1097.
- Berry S, Dean C. 2015.** Environmental perception and epigenetic memory: mechanistic insight through *FLC*. *Plant J* **83**: 133-148.
- Blázquez MA, Ahn JH, Weigel D. 2003.** A thermosensory pathway controlling flowering time in *Arabidopsis thaliana*. *Nat Genet* **33**: 168-171.
- Bratzel F, Turck F. 2015.** Molecular memories in the regulation of seasonal flowering: from competence to cessation. *Genome Biol* **16**: 192.
- Cassin J, Bourdeuet J, Fougue A, Furon V, Gaillard JP, Bourdelles P, Montaguad G, Moreuil C. 1969.** The influence of climate upon the blooming of citrus in tropical areas. *Proc. First Int. Citrus Symp.* **1**: 315-323.
- Charnov EL, Schaffer WM. 1973.** Life-History consequences of natural selection: Cole's result revisited. *Am. Naturalist* **107**: 791-793.
- Chica EJ, Albrigo LG. 2013.** Expression of flower promoting genes in sweet orange during floral inductive water deficits. *J. Am. Soc. Hort. Sci* **138**: 88-94.
- Clough SJ, Bent AF. 1998.** Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* **16**: 735-743.
- Cole LC. 1954.** The population consequences of life history phenomena. *Q Rev Biol* **29**: 103-137.
- Deng W, Ying H, Helliwell CA, Taylor JM, Peacock WJ, Dennis ES. 2011.** *FLOWERING LOCUS C (FLC)* regulates development pathways throughout the life cycle of *Arabidopsis*. *Proc Natl Acad Sci U S A* **108**: 6680-6685.
- Du J, Johnson LM, Jacobsen SE, Patel DJ. 2015.** DNA methylation pathways and their crosstalk with histone methylation. *Nat Rev Mol Cell Biol* **16**: 519-532.
- Earley KW, Haag JR, Pontes O, Opper K, Juehne T, Song K, Pikaard CS. 2006.** Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J* **45**: 616-629.
- Finnegan EJ, Dennis ES. 2007.** Vernalization-induced trimethylation of histone H3 lysine 27 at *FLC* is not maintained in mitotically quiescent cells. *Curr Biol* **17**: 1978-1983.

- Accepted Article
- Forment J, Gadea J, Huerta L, Abizanda L, Agusti J, Alamar S, Alos E, Andres F, Arribas R, Beltran JP, et al. 2005. Development of a citrus genome-wide EST collection and cDNA microarray as resources for genomic studies. *Plant Mol Biol* **57**: 375-391.
- Gramzow L, Theissen G. 2015. Phylogenomics reveals surprising sets of essential and dispensable clades of MIKC(c)-group MADS-box genes in flowering plants. *J Exp Zool B Mol Dev Evol* **324**: 353-362.
- Hartmann U, Hohmann S, Nettesheim K, Wisman E, Saedler H, Huijser P. 2000. Molecular cloning of *SVP*: a negative regulator of the floral transition in *Arabidopsis*. *Plant J* **21**: 351-360.
- Hou XJ, Liu SR, Khan MRG, Hu CG, Zhang JZ. 2014. Genome-wide identification, classification, expression profiling, and SSR marker development of the MADS-box gene family in *Citrus*. *Plant Mol Biol Rep* **32**: 28-41.
- Jean Finnegan E, Kovac KA, Jaligot E, Sheldon CC, James Peacock W, Dennis ES. 2005. The downregulation of *FLOWERING LOCUS C (FLC)* expression in plants with low levels of DNA methylation and by vernalization occurs by distinct mechanisms. *Plant J* **44**: 420-432.
- Jia H, Wang N. 2014. Targeted genome editing of sweet orange using Cas9/sgRNA. *PLoS ONE* **9**: e93806.
- Jones PA. 2012. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* **13**: 484-492.
- Lee J, He K, Stolc V, Lee H, Figueroa P, Gao Y, Tongprasit W, Zhao H, Lee I, Deng XW. 2007. Analysis of transcription factor HY5 genomic binding sites revealed its hierarchical role in light regulation of development. *Plant Cell* **19**: 731-749.
- Liebig GF, Chapman HD. 1963. The effect of variable root temperatures on the behavior of young navel orange trees in a green house. *Proc. Am. Soc. Hortic. Sci.* **82**: 204-209.
- Martinez-Alcantara B, Iglesias DJ, Reig C, Mesejo C, Agusti M, Primo-Millo E. 2015. Carbon utilization by fruit limits shoot growth in alternate-bearing citrus trees. *J Plant Physiol* **176**: 108-117.
- Martinez-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). *J Sci Food Agric* **90**: 1936-1943.
- Moss, GI. 1969. Influence of temperature and photoperiod on flower induction and inflorescence development in sweet orange. *J Hortic Sci* **44**: 311-320
- Munoz-Fambuena N, Mesejo C, Gonzalez-Mas MC, Primo-Millo E, Agusti M, Iglesias DJ. 2011. Fruit regulates seasonal expression of flowering genes in alternate-bearing 'Moncada' mandarin. *Ann Bot* **108**: 511-519.
- Nelson JD, Denisenko O, Sova P, Bomsztyk K. 2006. Fast chromatin immunoprecipitation assay. *Nucleic Acids Res* **34**: e2.
- Nishikawa F, Endo T, Shimada T, Fujii H, Shimizu T, Omura M, Ikoma Y. 2007. Increased *CiFT* abundance in the stem correlates with floral induction by low temperature in Satsuma mandarin (*Citrus unshiu* Marc.). *J Exp Bot* **58**: 3915-3927.
- Pien S, Fleury D, Mylne JS, Crevillen P, Inze D, Avramova Z, Dean C, Grossniklaus U. 2008. *ARABIDOPSIS TRITHORAX1* dynamically regulates *FLOWERING LOCUS C* activation via histone 3 lysine 4 trimethylation. *Plant Cell* **20**: 580-588.
- Reeves PA, He Y, Schmitz RJ, Amasino RM, Panella LW, Richards CM. 2007. Evolutionary conservation of the *FLOWERING LOCUS C*-mediated vernalization response: evidence from the sugar beet (*Beta vulgaris*). *Genetics* **176**: 295-307.

- Ruelens P, de Maagd RA, Proost S, Theissen G, Geuten K, Kaufmann K. 2013.** *FLOWERING LOCUS C* in monocots and the tandem origin of angiosperm-specific MADS-box genes. *Nat Commun* **4**: 2280.
- Sgamma T, Jackson A, Muleo R, Thomas B, Massiah A. 2014.** *TEMPRANILLO* is a regulator of juvenility in plants. *Sci Rep* **4**: 3704.
- Shalom L, Samuels S, Zur N, Shlizerman L, Zemach H, Weissberg M, Ophir R, Blumwald E, Sadka A. 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.
- Sheldon CC, Rouse DT, Finnegan EJ, Peacock WJ, Dennis ES. 2000.** The molecular basis of vernalization: the central role of *FLOWERING LOCUS C* (*FLC*). *Proc Natl Acad Sci U S A* **97**: 3753-3758.
- Suarez-Lopez P, Wheatley K, Robson F, Onouchi H, Valverde F, Coupland G. 2001.** *CONSTANS* mediates between the circadian clock and the control of flowering in *Arabidopsis*. *Nature* **410**: 1116-1120.
- Tamada Y, Yun JY, Woo SC, Amasino RM. 2009.** *ARABIDOPSIS TRITHORAX-RELATED7* is required for methylation of lysine 4 of histone H3 and for transcriptional activation of *FLOWERING LOCUS C*. *Plant Cell* **21**: 3257-3269.
- Wang R, Farrona S, Vincent C, Joecker A, Schoof H, Turck F, Alonso-Blanco C, Coupland G, Albani MC. 2009.** *PEP1* regulates perennial flowering in *Arabis alpina*. *Nature* **459**: 423-427.
- Wells CE, Vendramin E, Jimenez Tarodo S, Verde I, Bielenberg DG. 2015.** A genome-wide analysis of MADS-box genes in peach [*Prunus persica* (L.) Batsch]. *BMC Plant Biol* **15**: 41.
- Whittaker C, Dean C. 2017.** The *FLC* locus: a platform for discoveries in epigenetics and adaptation. *Annu Rev Cell Dev Biol* **33**: 555-575.
- Yang H, Howard M, Dean C. 2014.** Antagonistic roles for H3K36me3 and H3K27me3 in the cold-induced epigenetic switch at *Arabidopsis FLC*. *Curr Biol* **24**: 1793-1797.
- Zhou CM, Zhang TQ, Wang X, Yu S, Lian H, Tang H, Feng ZY, Zozomova-Lihova J, Wang JW. 2013.** Molecular basis of age-dependent vernalization in *Cardamine flexuosa*. *Science* **340**: 1097-1100.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 *CcMADS19* is an *FLC* ortholog.

Fig. S2 Time-course expression of flowering related genes.

Fig. S3 Diagrammatic representation of the epigenetic regulation of *CcMADS19*, the endogenous and exogenous control of *CiFT2*, and bud prouting and flowering during three consecutive seasons in *Citrus* grown under Mediterranean climate.

Table S1 Primer sequences used in this study.

Table S2 Position of CG and CHH analysed.

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Author contribution

MA, CM, EPM and MB planned and designed the research; NMF, AMF, CR and DJI performed experiments and conducted fieldwork; FVS, MdL, AMF and CR carried out biochemical analyses; MA, CM, MdL, EPM and MB analysed data; and MA, CM and MB wrote the manuscript. MA, MB and CM contributed equally.

Figure Legends

Fig. 1 Time-course of alternate bearing in *Citrus* trees during 4 consecutive seasons. The high number of flowers on year 1 (ON year) (a) gave rise to a large crop (b), and it reduced dramatically the following bloom and yield on year 2 (OFF year), which, in turn, allows a high flowering and yield on year 3, and so on. The OFF year, therefore, begins with an absence of flowers and high vegetative sprouting in spring, contrary to what happens in the ON year, that sprouts 5 times lower, in our experiment (c); the sprouting of autumn having similar behaviour. Consequently, during the floral bud inductive period (November-December) the ON trees are loaded with fruit and have hardly any new vegetative development, while the OFF trees have only been vegetatively developed and have no fruit (d). The experiment was carried out with 12 trees, 6 ON- and 6 OFF-year tree, of the highly alternate bearing 'Moncada' mandarin [*C. clementina* x (*C. unshiu* x *C. nobilis*)]. SE are giving as vertical bars (n = 6).

Fig. 2 Average minimum temperature (a) and expression pattern of the *CiFT2* (b) and *CcMADS19* (c) genes on leaves of ON and OFF trees of 'Moncada' mandarin [*C. clementina* x (*C. unshiu* x *C. nobilis*)] throughout a year. Values are referred to gene expression in ON trees in May. Data are the mean of three biological replicates and two technical replicates each. Data are mean \pm SE (n = 3).

Fig. 3 Flowering intensity (a), and *CiFT2* (b), and *CcMADS19* (c) genes relative expression in leaves of ON, OFF and DEF trees (ON-defruited trees) of 'Moncada' mandarin [*C. clementina* x (*C. unshiu* x *C. nobilis*)]. Gene expression was analysed in leaves sampled at the floral bud inductive period (November the 30), and flowering was evaluated in spring of the following season. Defruiting was carried out in July, just after fruit set. Data are mean \pm SE. Different letters indicate differences in a Student's t-test ($p \leq 0.05$, n = 6).

Fig. 4 *CcMADS19* represses flowering in *Arabidopsis* Col-0 accession when expressed under a CaMV35S promoter. The homozygous transgenic plants delay flowering (a) and increase the number of rosette leaves (b). Asterisks indicate statistically significant differences with respect to the untransformed wild type ($p < 0.01$; n=50), and letters in (b) indicate differences in ANOVA test (n=50). (c) *CcMADS19* reduces *CiFT2* gene expression in *Citrus* leaves from OFF trees when agroinfiltrated with *A. tumefaciens* carrying the 35S::*CcMADS19* construct

16h after infection with *Pseudomonas syringae* (*P. s.*, 10^4 and 10^8 CFU/ml). Two days later, *CiFT2* gene expression was determined by RT-qPCR. Leaves were sampled from OFF trees of 'Moncada' mandarin [*C. clementina* x (*C. unshiu* x *C. nobilis*)] at the floral bud inductive period (November 30); n = 50. Data are mean \pm SE. Asterisks indicate statistical significance in a Student's t-test ($p < 0.01$, n=5).

Fig. 5 DNA methylation profiles of *CcMADS19* locus (a). Colored bars show the percentage of cytosine methylation (mC). Bisulphite sequencing was performed on DNA collected from leaves of 'Afourer' tangor (*C. reticulata* x *C. sinensis*) ON trees, OFF trees, and trees defruited in the summer (DEF), at the floral bud inductive period (30 November). Black dots mark the positions with statistically significant differential behavior between ON and DEF/OFF trees. Statistical significance was calculated with Fisher's exact test ($n \geq 10$, $p < 0.05$). (b) Effect of 5-azacytidine (5-aza, 350 μ M) applied at the floral bud inductive period (November 25) on the relative expression levels of *CcMADS19* and *CiFT2* in the leaves of single flowered leafy shoots of 'Afourer' tangor. Treatment was applied as a foliar spray. Data are means of 5 trees and 3 biological replicates. Data are mean \pm SE. Asterisks indicate statistical significance in a Student's t-test ($p < 0.01$, n=5)

Fig. 6 *CcMADS19* active/repressed state correlates with changes in histone methylation. (a) H3K4me3 levels in leaves determined by ChIP of two regions located on the promoter of the *CcMADS19* locus. (b) Relative expression in leaves of methyltransferases *ATX1-like* and *ATX7-like* genes determined by RT-qPCR. Data correspond to 'Afourer' tangor (*C. reticulata* x *C. sinensis*) leaves from ON and OFF trees, and ON trees defruited in the summer (DEF), sampled at the floral bud inductive period (30 November). (c) H3K27me3 levels in buds determined by ChIP of two regions located on the promoter of the *CcMADS19* locus. (d) Relative expression in buds and leaves of *CcMADS19*. Data correspond to lateral buds from ON and OFF trees of 'Afourer' tangor sampled at floral bud differentiation (15 February). Data are mean \pm SE. Asterisks indicate statistical significance in a Student's t-test ($p < 0.01$, n=3). ns: not significant difference.









