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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 lifetime, alternating vegetative and reproductive meristems in the same individual.
- To understand how fruits regulate flowering in polycarpic plants, we focused on alternate bearing in *Citrus* trees that had been experimentally established as fully flowering or nonflowering.
- We 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. By contrast, newly emerging shoots display an opposite epigenetic scenario associated with *CcMADS19* repression, thereby allowing the activation of *CiFT2* the following cold season.

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 lifetime, and itelparity 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 itelparity 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 unfavourable conditions by blocking the response to inductive signals by the MADS domain transcription factor *FLOWERING LOCUS C* (*FLC*) and its homologues 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* orthologue *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 the plant reaches a certain age, 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, such as 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 the autumn 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 in the expression of the *Citrus* orthologue of *FT* (*CiFT2*) (Nishikawa *et al.*, 2007; Chica & Albrigo, 2013). Interestingly, fruit remaining on the tree during the floral bud inductive period is correlated 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 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 solely 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-yr-old trees of 'Moncada' mandarin (Clementina Oroval (*Citrus clementina Hort.* ex *Tan.*) × 'Kara' mandarin (*C. unshiu* Marc. × *C. nobilis* Lou.)) and 12-yr-old 'Afourer' tangor (*Citrus reticulata* × *Citrus sinensis*), grafted onto Carrizo citrange (*C. sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.) rootstock, and exhibiting a marked alternate bearing. Trees were planted 5 × 5 m apart, drip-irrigated, fertilized and grown according to usual techniques. The experimental field was located at the IVIA Research Station (Moncada, Spain). *Arabidopsis thaliana* seeds (Col-0) were surface-sterilized and grown in a growth room under 16 h : 8 h, light : dark cycle (light intensity = 150–200 μmols m^{-2} s^{-1}) 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 six ON (fully loaded) and six OFF (without fruit) trees randomly selected according their uniformity in size and vigour. 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 autumn, the number of vegetative shoots was counted from the same branches, with the results also quoted per 100 nodes. Total yield per tree was determined by weighing all fruits at harvest (February). Defruiting experiments were performed on another set of six 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 11:00 h for RNA extractions. In mid-January, 30 buds per each kind of tree were also sampled at 11:00 h 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 gene expression was studied on three ON trees treated three times (September, October and November). A nonionic surfactant Tween® 20 (polyethylene glycol sorbitan monolaurate; Sigma-Aldrich) at a concentration of 0.02% was added to the solution. Young leaves (2 months old) were sampled at 0, 24 and 48 h after the last treatment. Untreated trees were used as controls for comparison.

Sequence analysis

Amino acid sequences of the genes studied were obtained from the Phytozome v.10.3 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). 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 the manufacturer's instructions. cDNA was obtained from 1 μg total RNA using the QuantiTect® Reverse Transcription Kit (Qiagen) in a total volume of 20 μl. Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) was carried out on a Rotor Gene Q 5-Plex using the QuantiTect® SYBR® Green PCR Kit (both Qiagen). The reaction mix and conditions followed the manufacturer's instructions with certain modifications. The PCR mix contained 2.5 μl of a four-fold cDNA dilution, 12.5 μl of QuantiTect® SYBR Green PCR Master Mix, 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 preincubation, 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 Supporting Information Table S1.

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). The bisulphite treated DNA was amplified using Hot start *Platinum*® *Taq* DNA Polymerase (Invitrogen). Primer sequences are presented in Table S1. The thermal cycling programme was set at 95°C for 1 min followed by 40 cycles of 95°C for 30 s, annealing at 50°C 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 Table S1, 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 GenBank 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^{-1}) resuspended in sterile tap water (5×10^8 CFU ml^{-1}). Sixteen hours later, the same inoculated leaf areas were subjected to agroinfiltration as described previously. Recombinant *A. 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 $OD_{600} = 0.8$, the inoculum was harvested and resuspended in MMA solution (10 mM $MgCl_2$, 10 mM MES, pH 5.6, and 200 mM AS) to a final OD_{600} of 1.0.

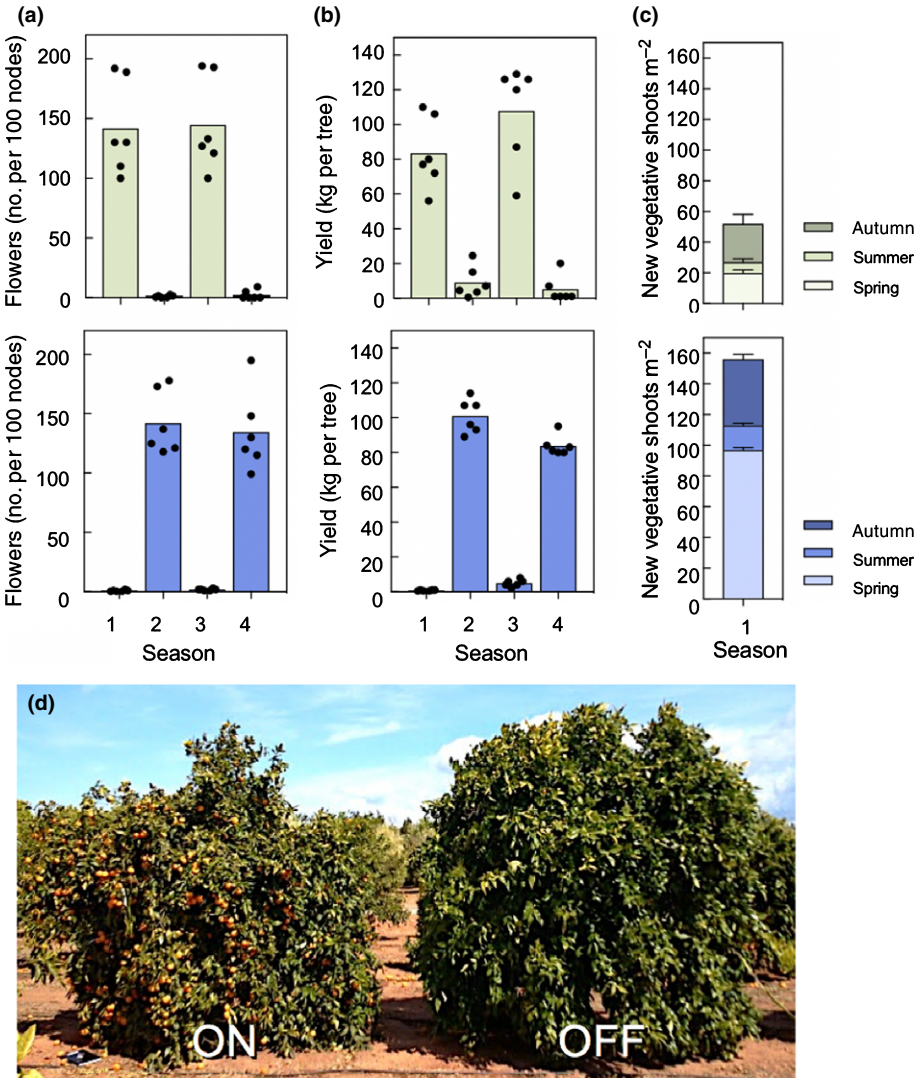

Fig. 1 Time-course of alternate bearing in *Citrus* trees during four consecutive seasons. The high number of flowers in year 1 (ON year – heavy fruit yield) (a) gave rise to a large crop (b), and it reduced dramatically the subsequent bloom and yield in year 2 (OFF year – no fruit production), which, in turn, allowed high flowering and yield in 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, with five-fold lower sprouting in our experiment (c), the sprouting in autumn showing 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, whereas the OFF trees have only been vegetatively developed and have no fruit (d). The experiment was carried out with 12 trees, six ON- and six OFF-year trees, of the highly alternate bearing mandarin cv Moncada (*C. clementina* × (*C. unshiu* × *C. nobilis*)). SE is shown as vertical bars ($n = 6$).

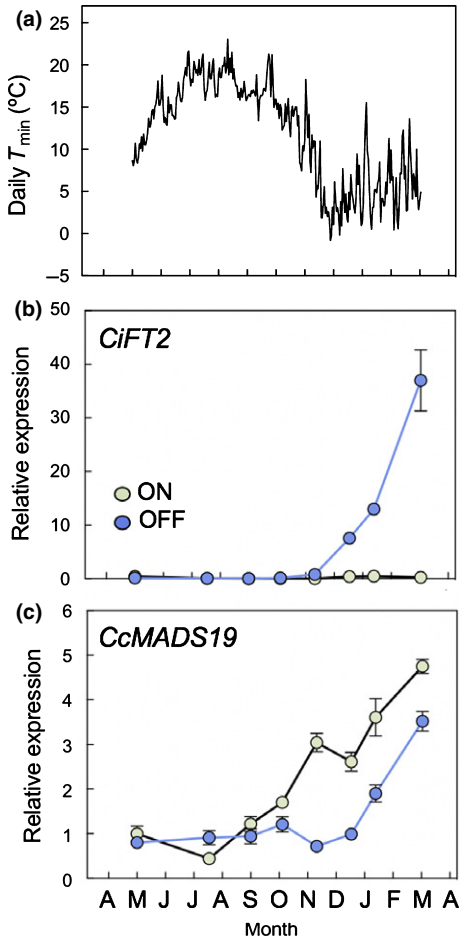

Fig. 2 Average minimum temperature (T_{min}) (a) and expression pattern of the *CiFT2* (b) and *CcMADS19* (c) genes on leaves of ON (heavy fruit yield) and OFF (no fruit production) trees of mandarin cv Moncada (*C. clementina* × (*C. unshiu* × *C. nobilis*)) throughout a year. Values are referred to gene expression in ON trees in May. Data are the means of three biological replicates and two technical replicates each. Data are means ± SE ($n = 3$).

The suspension was left at room temperature for 2 h and infiltrated in the same area previously inoculated with *P. syringae*. Citrus leaves agroinfiltrated with *Agrobacterium* in the absence of *P. syringae* inoculation were used as controls. The presence of the agroinfiltrated protein was confirmed by Western blotting.

Chromatin inmunoprecipitation

Chromatin inmunoprecipitation (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 (Woburn, MA, USA) 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 h, and chromatin-antibody complexes were captured with protein A/G magnetic beads (Thermo Fisher Scientific, Waltham, MA, USA). De-crosslinking reaction was performed with Chelex slurry (Bio-Rad, Watford, UK) as previously described (Nelson *et al.*, 2006).

For the ideintification of the H3K27me3- and H3K4me3-regulated regions, we first divided the *CcMADS19* promoter (5000 bp) and genomic region (13 800 bp) in bins of 1000 bp, and designed primers to amplify *c.* 180 bp within each bin. Nineteen pairs of primers were screened in total by quantitative PCR against the input. We then performed a comparative analysis between induced and noninduced samples.

Results and Discussion

CcMADS19 gene expression correlates with fruit-mediated flowering inhibition

Citrus trees of the mandarin cv Moncada maintain marked alternate bearing (Munoz-Fambuena *et al.*, 2011). The 12 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, that is, 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 produced barely 10 kg (Fig. 1b). Both groups of trees maintained alternate bearing behaviour during the 4 yr of the experiment. Reciprocally, trees in the ON state produced only 53 vegetative shoots m^{-2}, whereas OFF trees reached over 160 vegetative shoots m^{-2} during the spring, summer and autumn flushes (Fig. 1c,d).

Although the orthologues 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* orthologues appear indistinctly in some species (Fig. S1a,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 *C. sinensis* and *C. 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 whether the *FLC* orthologue 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 the spring in OFF trees in response to low temperature, which promotes flowering (Moss, 1969; Nishikawa *et al.*, 2007), but not in ON trees (Fig. 2a,b). Interestingly, this effect was inversely correlated with the expression of *CcMADS19*, which was higher in ON than in OFF trees at the moment when the floral transition was established in OFF trees, that is, 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;

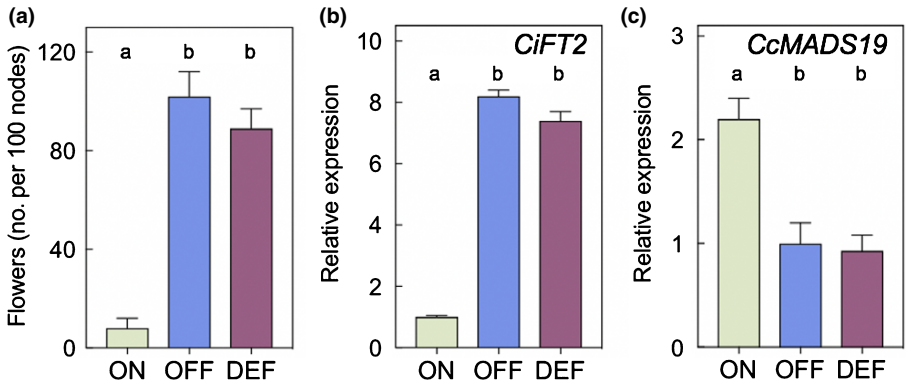

Fig. 3 Flowering intensity (a), and relative expression of *CiFT2* (b) and *CcMADS19* (c) genes in leaves of ON (heavy fruit yield), OFF (no fruit production) and DEF (defruited ON) trees of mandarin cv Moncada (*C. clementina* × (*C. unshiu* × *C. nobilis*)). Gene expression was analysed in leaves sampled at the floral bud inductive period (30 November), and flowering was evaluated in the spring of the following season. Defruiting was carried out in July, just after fruit set. Data are means ± SE. Different letters indicate differences in a Student's *t*-test ($P \leq 0.05$, $n = 6$).

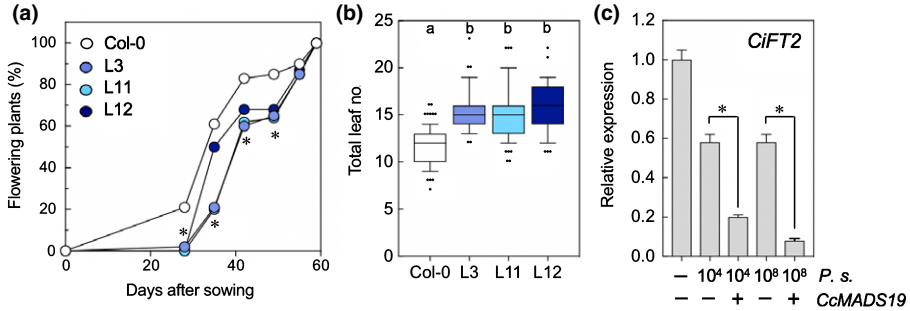

Fig. 4 *CcMADS19* represses flowering in *Arabidopsis* Col-0 accession when expressed under a CaMV35S promoter. (a, b) The homozygous transgenic plants delay flowering (a) and increase the number of rosette leaves (b). Asterisks indicate statistically significant differences with respect to the untransformed 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 (no fruit production) trees when agroinfiltrated with *Agrobacterium tumefaciens* carrying the *35S::CcMADS19* construct 16 h after infection with *Pseudomonas syringae* (*P.s.*, 10^4 and 10^8 CFU ml^{-1}). Two days later, *CiFT2* gene expression was determined by quantitative reverse transcription polymerase chain reaction. Leaves were sampled from OFF trees of mandarin cv Moncada (*C. clementina* × (*C. unshiu* × *C. nobilis*)) at the floral bud inductive period (30 November) ($n = 50$). Data are means ± SE. Asterisks indicate statistical significance in a Student's *t*-test ($P < 0.01$, $n = 5$).

Fig. 2a,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 vs OFF trees was specific to *CcMADS19*, as the expression in leaves of *CcMADS42* and *TEMPRANILLO-LIKE1* (*CcTEML1*), whose orthologues in Arabidopsis, *SHORT VEGETATIVE PHASE* (*SVP*) and T*EMPRANILLO1* (*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 1 yr period (Fig. S2a,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). Leaves of DEF trees showed similar *CiFT2* and *CcMADS19* gene expression to 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 among ON, OFF and DEF trees for *CcMADS42* and *CcTEML1* genes (Fig. S2c).

CcMADS19 is a floral repressor that downregulates *CiFT2* expression

The observations that *CcMADS19* is an orthologue of *FLC* (Hou *et al.*, 2014), that it displays a temporal expression pattern opposite to that of *CiFT2*, and has an expression level that 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 the number of rosette leaves significantly (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* orthologue (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 when 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 *TEM1*-like genes. Leaves

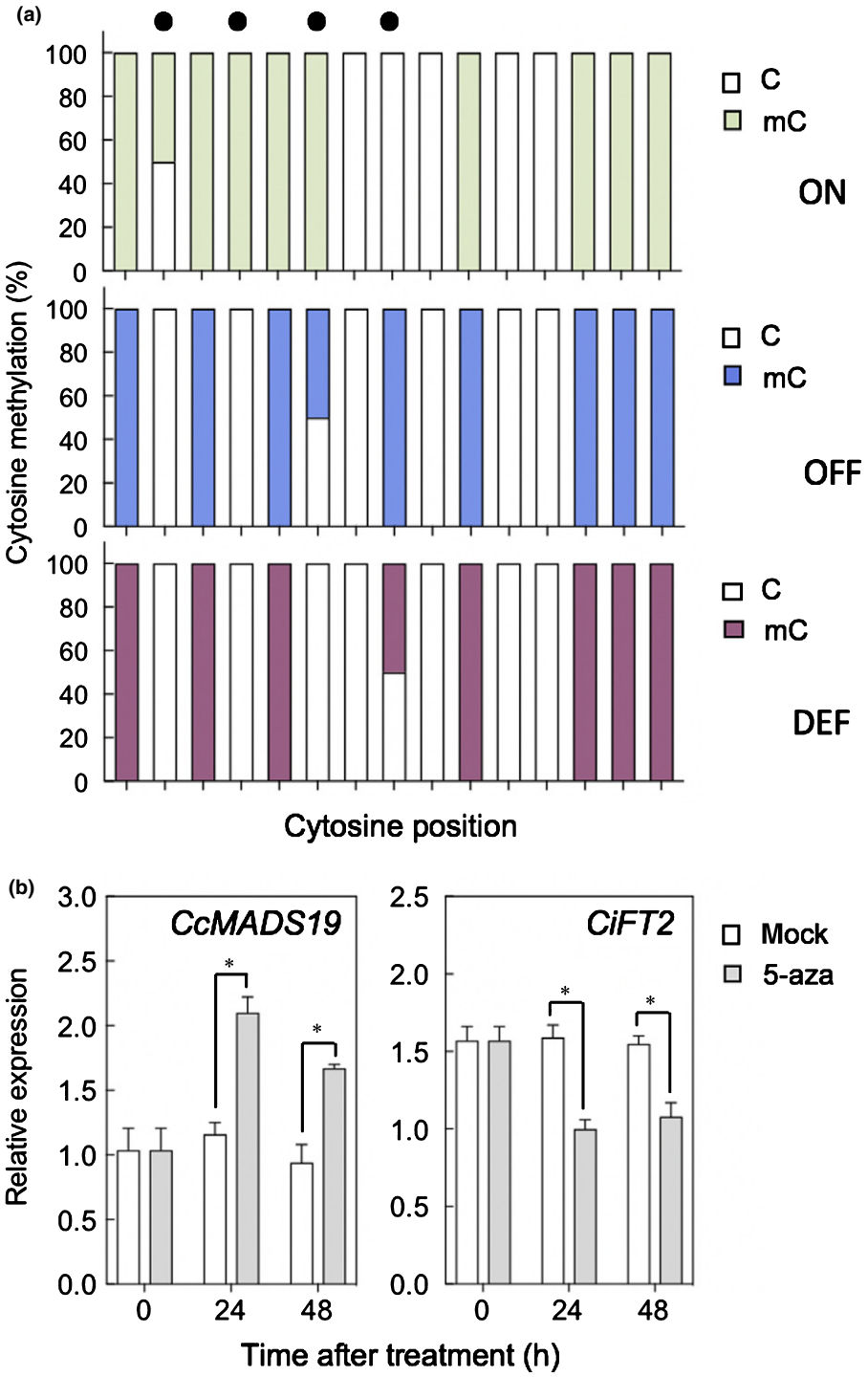

Fig. 5 (a) DNA methylation profiles of *CcMADS19* locus. Coloured bars show the percentage of cytosine methylation (mC). Bisulphite sequencing was performed on DNA collected from leaves of 'Afourer' tangor (*Citrus reticulata* × *Citrus sinensis*) ON trees (heavy fruit yield), OFF trees (no fruit production) and ON trees defruited in the summer (DEF), at the floral bud inductive period (30 November). Black dots mark the positions with statistically significant differential behaviour 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 μM) applied at the floral bud inductive period (25 November) 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 five trees and three biological replicates. Data are means ± SE. Asterisks indicate statistical significance in a Student's *t*-test ($P < 0.01$, $n = 5$)

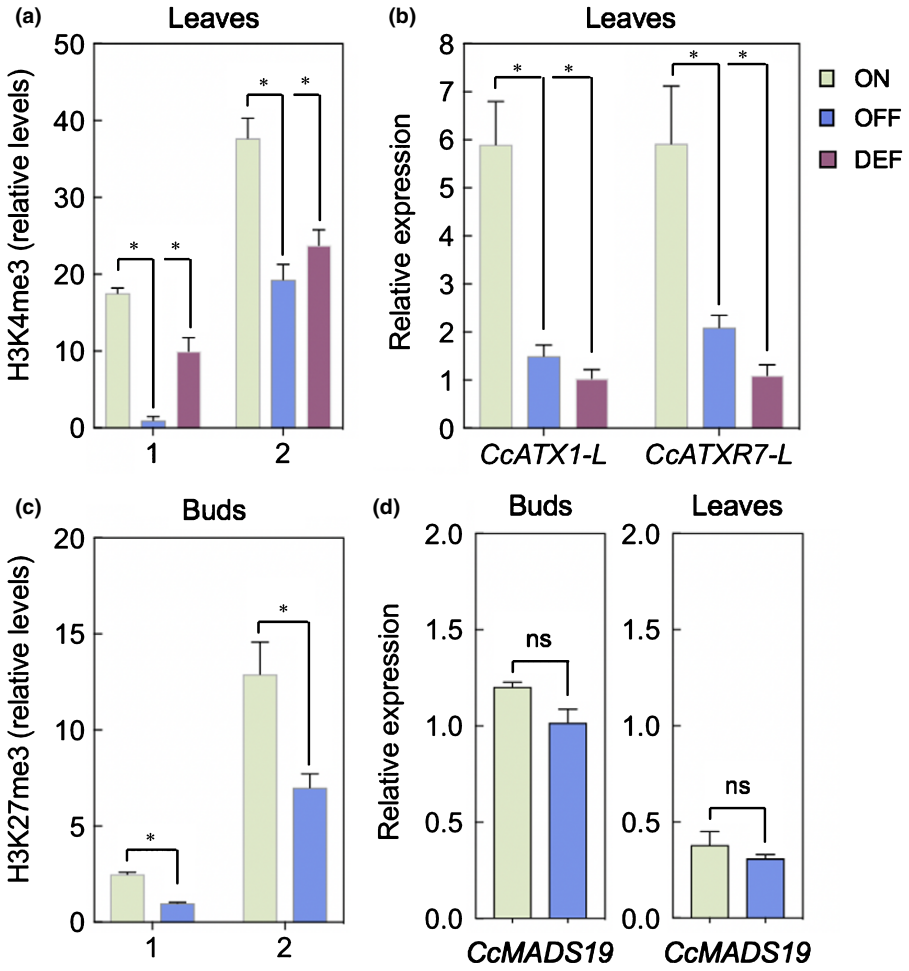

Fig. 6 *CcMADS19* active/repressed state is correlated with changes in histone methylation. (a) H3K4me3 levels in leaves determined by chromatin immunoprecipitation (ChIP) of two regions located on the promoter of the *CcMADS19* locus. (b) Relative expression in leaves of methyltransferase *ATX1-like* and *ATX7-like* genes determined by quantitative reverse transcription polymerase chain reaction. Data correspond to 'Afourer' tangor (*Citrus reticulata* × *Citrus sinensis*) leaves from ON (heavy fruit yield) and OFF trees (no fruit production), 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 means ± SE. Asterisks indicate statistical significance in a Student's *t*-test ($P < 0.01$, $n = 3$). ns, nonsignificant difference.

were sampled at the floral inductive period (November), when *CcMADS19* is differentially expressed in ON and OFF trees (see Fig. 2c). Although no difference in cytosine methylation pattern was found between ON and OFF trees in the seven, eight and 20 CG sites of *CiFT2*, *CcMADS42* and *CcTEML1*, respectively (Table S2), we did find significant changes in cytosine methylation in the *CcMADS19* gene. Methylation was examined in three regions (Fig. S1c): the proximal promoter (−1000 bp); intron 1, from +8035 to +8421 bp; and intron 1, from +8858 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 (four out of 10 clones) in OFF trees (Table S2). 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) (Table S2). More importantly, DEF trees rendered a methylation pattern that was 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 on *CcMADS19* and *CiFT2* expression. This chemical is a cytosine analogue which inhibits DNA methyltransferases and modifies cytosine methylation and gene expression (Chang & Pikaard, 2005). In the ON leaves treated with 5-azacytidine, *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 states of the *FLC* and *PEP1* genes, respectively, are correlated 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 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, that is, 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 orthologues of the methlyltransferases *TRITHORAX 1* (*TRX1*) and *TRX7*, required for the activation of *FLC* expression in Arabidopsis (Pien *et al.*, 2008; Tamada *et al.*, 2009), were correlated with the level of the H3K4me3 mark in ON, OFF and DEF trees (Fig. 6b).

These results suggest that the presence of the fruit provokes the epigenetic activation of *CcMADS19* in the adjacent mature leaves, to repress, locally and temporally, *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 a 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 (Fig. S3). This mechanism resembles the seasonal vernalization switch in perennial herbaceous species, such as *A. alpina*, or the generational switch occurring during meiosis in annual species, such as *A. thaliana*. However, it is important to note 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, such as the life span of reproductive meristems in annual species, although in that case, shoot apical meristem-specific genes are irreversibly shut off (Balanza *et al.*, 2018). To understand whether equivalent signals regulate both processes still requires further study.

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Author contributions

MA, CM, EP-M and MAB planned and designed the research; NM-F, AM-F, CR and DJI performed experiments and conducted the fieldwork; FV-S, MdL, AM-F and CR carried out biochemical analyses; MA, CM, MdL, EP-M and MAB analysed the data; and MA, CM and MB wrote the manuscript. MA, MAB and CM contributed equally to this work.

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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* orthologue.

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 sprouting and flowering during three consecutive seasons in *Citrus* grown in a Mediterranean climate.

Table S1 Primer sequences used in this study.

Table S2 Position of CG and CHH analysed.

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