Research

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Reversion of fruit-dependent inhibition of flowering in *Citrus* requires sprouting of buds with epigenetically silenced *CcMADS19*

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Summary

- In *Citrus*, the response to environmental floral inductive signals is inhibited by the presence of developing fruits. The mechanism involves epigenetic activation of the *CcMADS19* locus (*FLC* orthologue), encoding a floral repressor.
- To understand how this epigenetic regulation is reverted to allow flowering in the following season, we have forced precocious sprouting of axillary buds in fruit-bearing shoots, and examined the competence to floral inductive signals of old and new leaves derived from them.
- We have found that *CcMADS19* is enriched in repressive H3K27me3 marks in young, but not old leaves, revealing that axillary buds retain a silenced version of the floral repressor that is mitotically transmitted to the newly emerging leaves, which are able to induce flowering.
- Therefore, we propose that flowering in *Citrus* is necessarily preceded by vegetative sprouting, so that the competence to respond to floral inductive signals is reset in the new leaves.

Introduction

Plants growing in temperate climates align their reproductive cycles with the seasons. The optimal time of flowering is established through the integration of endogenous and exogenous information, which is remembered through a mechanism that converges on the dynamic deposition and removal of epigenetic marks in key integrator genes (reviewed in Andrés & Coupland, 2012 and Bratzel & Turck, 2015). For instance, in annual plants that require vernalisation to flower, such as *Arabidopsis thaliana*, cold temperature triggers flowering through epigenetic silencing of a floral repressor gene, *FLOWERING LOCUS* C (*FLC*) (Finnegan & Dennis, 2007). This epigenetic repression is established by the Polycomb Repressive Complex 2 (PRC2) through trimethylation in Histone 3 Lys 27 (H3K27me3), which is targeted to the *FLC* locus by the *VERNALIZATION INSENSITIVE3* (*VIN3*) homeodomain transcription factor (De Lucía *et al.*, 2008). A critical step in this process is the restoration of the vernalisation requirement in the next generation. This is achieved during meiosis in the anthers, through epigenetic reprogramming of *FLC* by the jumonji-containing ELF6 protein, which directs H3K27me3 demethylation in gametes (Crevillén *et al.*, 2014).

In polycarpic plants, such as *Arabis alpina*, the *FLC* orthologue named *PERPETUAL FLOWERING1* (*PEP1*) causes default repression of flowering in a similar mechanism to the one proposed for *FLC* (Wang *et al.*, 2009). However, the degree of *PEP1* stable silencing by vernalisation varies between meristems, therefore *PEP1* expression is reactivated during spring in some axillary meristems to promote vegetative growth and contribute to the polycarpic habit (Wang *et al.*, 2009; Lázaro *et al.*, 2018). This spatial difference between meristems indicates the key role of endogenous factors in the flowering process.

A particular case of endogenous control of flowering is observed in some cultivars of both evergreen and deciduous fruit tree species such as apple, avocado, mango, olive, pecan and pistachio, among others, and also in *Citrus*, in which the response to environmental floral inductive signals (i.e. cold temperature) is locally gated by the presence of developing fruits. Therefore, the fruits initiated in the previous season (ON season) block flowering in the following season (OFF season), and lead to a phenomenon called *alternate bearing* (Tuckey, 1922; Monselise & Goldschmidt, 1982; Goldschmidt & Sadka, 2021). As for *FLC* and *PEP1*, the fruit-mediated inhibition of flowering observed in

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Citrus is associated to epigenetic changes in *CcMADS19* (the *Citrus clementina FLC* orthologue; Hou *et al.*, 2014). Namely, the presence of the fruit is paralleled by (1) an enrichment of H3K4me3 mark in the *CcMADS19* locus in the neighbouring leaves, (2) induction of *CcMADS19* expression, and (3) subsequent repression of the *Citrus FT* orthologue (*CiFT3*, Ciclev10012905m, previously named *CiFT2*), during the floral induction period (Agustí *et al.*, 2020). Although this mechanism explains the inhibition of flowering by fruits, it does not explain the necessary reprogramming that allows ON trees to flower in the next (OFF) season (Hoijemberg & Cerdán, 2020).

In the present study, we established the hypothesis that the epigenetic activation of *CcMADS19* in the leaf during the ON season is reset in the axillary buds, and, through cell divisions, transmitted to the new emerging leaves which then become competent to flower in the next OFF season. Our hypothesis is based on the following observations: (1) *CcMADS19* expression in the buds from ON trees is low just before budbreak (Agustí *et al.*, 2020); (2) these buds show enrichment of H3K27me3 in the *CcMADS19* locus (Agustí *et al.*, 2020); and (3) flowering in the OFF season is preceded by extensive sprouting and vegetative development of previously dormant buds (Verreyne & Lovatt, 2009).

To test the above hypothesis, we used girdling, an experimental approach that induces precocious vegetative development in the axillary buds during the ON season before the cold inductive period (Agustí *et al.*, 1992). This way, we can compare the effect of the floral inductive signals between old leaves and the new leaves derived from the buds in the presence of fruits.

Materials and Methods

Plant material and growth conditions

Experiments were carried out using field grown 10-yr-old trees of 'Nadorcott' mandarin (*Citrus clementina* Hort. *ex* Tan. × *C. sinensis* (L.)), grafted onto Carrizo citrange (*C. sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.) rootstock, and exhibiting a marked alternate bearing. Trees were planted 6 m × 4 m apart, drip irrigated, fertilised and grown according to usual techniques. The experiment was carried out in a commercial orchard located in València (Spain).

Experimental design and tree phenotyping

The effect of fruit load on flowering was studied on 4 ON (fully loaded) and 4 OFF (without fruit) trees randomly selected according their uniformity in size and vigour. In a set of 16 ON trees, a defruiting experiment was performed by removing 0%, 33%, 66% and 100% of the developing fruits per tree at the onset of stage II of fruit development (July).

Flowering intensity was evaluated in early spring a few days before anthesis. Four branches distributed uniformly around the tree having some 300 nodes per branch were selected, and the number of sprouted nodes, shoots initiated and flowers per shoot were counted. From the number of shoots developed and the number of flowers per shoot, the total number of flowers could be calculated. The results were expressed in shoots and flowers per 100 nodes to compensate for the differences in size of the branches selected for counting. In summer and fall, vegetative shoots developed from the same branches were also counted, referring the results also per 100 nodes. Total yield per tree was determined by weighing all fruits at harvest (February).

Girdling experiments were performed in 200 leafy single flowered shoots (ON) and vegetative shoots (OFF) of 10 trees. ON shoots are determinate inflorescences with an apical fruit and 6–8 nodes along 15–25 cm which contain the axillary buds. Girdling was performed by removing a 2-mm ring of bark from the peduncle 1 cm away from the calyx (Supporting Information Fig. S1). The effect of the date of girdling (early summer, late summer and early autumn) on sprouting and flowering was also assayed. Fruits from ON shoots that were not girdled served as controls. The days following girdling and in the next spring, axillary bud sprouting and flowering were evaluated in 50 shoots per treatment. The experiment was repeated in 2 yr.

Buds were sampled 7 d after girdling for IAA analysis by UPLC-MS/M. From October to January, leaves from ON, OFF and ON-girdled shoots were collected for RNA extractions. Finally, the new shoots produced as a result of girdling were sampled in September for chromatin immunoprecipitation analysis. All samples were immediately ground and stored at −80°C until analysed.

Sequence analysis

Amino acid sequences of the genes studied were obtained from the PHYTOZOME v.13 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 OD_{260}/OD_{280} ratio and gel electrophoresis. RNA concentration was determined by fluorometric assays with the RiboGreen dye (Molecular Probes, Eugene, OR, USA) according to manufacturer's instructions. 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) using the QuantiTect® SYBR® Green PCR Kit (Qiagen). 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), 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 Table S1.

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) for 4 h, and chromatin–antibodies complexes were captured with protein A/G magnetic beads (Thermo Scientific). De-crosslinking reaction was performed with Chelex slurry (Bio-Rad) as described (Nelson *et al.*, 2006).

For the identification of the H3K27me3 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. In total, 19 pairs of primers were screened by qPCR against the input. We then performed a comparative analysis between induced and noninduced samples.

Statistical analysis

STATGRAPHICS PLUS software was used to analyse the data. Analysis of variance (ANOVA) was performed using Fisher's least significant difference test for mean separations at $P < 0.05$.

Results and Discussion

Restarting vegetative growth in ON shoots allows flowering

Under temperate climate conditions, *Citrus* trees flower and set fruits only once a year, in spring. But they sprout again in summer–autumn, only producing vegetative shoots. This latter sprouting is important because floral shoots of the next season in spring develop mainly on these vegetative shoots (Guardiola *et al.*, 1982). It has been proposed that the presence of fruits not only represses flowering but also vegetative sprouting (Verreynne & Lovatt, 2009; Martínez-Fuentes *et al.*, 2010). Accordingly, we observed that early fruit removal (July) significantly increased bud sprouting in summer–autumn and next spring, and also flowering, the intensity of which depended on the defruiting intensity (Fig. 1a,b). Interestingly, control trees (i.e. ON) were able to only produce five shoots per 100 nodes in summer/fall, and 17 in spring (Fig. 1a), bearing practically no flowers (Fig. 1b). Conversely, thinning 66% and 100% of fruits increased summer–autumn sprouting up to eight and 18 shoots per 100 nodes, respectively (Fig. 1a), and significantly boosted flowering (Fig. 1b). In summary, during an ON season the axillary bud sprouting is very low, and its flowering-ability results are abolished due to the epigenetic activation of the *CcMADS19* gene in the leaves (Agustí *et al.*, 2020).

To further understand the role of sprouting in the resetting of the flowering ability after the ON season, and *CcMADS19* reprogramming, we focused on the short-distance effect caused by the fruit to the lateral buds. Citrus trees set fruits in the apical position of determinate inflorescences, and girdling reverts the inhibitory effect on proximal bud sprouting (Martínez-Fuentes *et al.*, 2010). In fact, girdling or ringing branches in midsummer is used to mitigate biennial bearing when performed in the OFF year (Agustí *et al.*, 1992; Goren *et al.*, 2010). Accordingly, fruit-peduncle girdling in early (G1) and late (G2) summer allowed sprouting of the first three or four nodes of the eight nodes closest to the girdle (Fig. 2). Moreover, it has been proposed that sprouting inhibition is mediated by the outbound flux of auxin from the fruit (Bangerth *et al.*, 2000; Krasniqi *et al.*, 2013). Consequently, the girdling-promoted sprouting correlated with a decrease in auxin levels in the shoot (Fig. S2), which is in agreement with the report that girdling impairs polar auxin transport (Ferguson & Beveridge, 2009). A few days after girdling in early and late summer, lateral buds sprouted, achieving 22% in the second date of treatment (G2), whereas ON shoots girdled in the third date (G3, early autumn), and ungirdled ON shoots did not sprout. Nonbearing branches (OFF) sprouted naturally up to 27% in July and September (Fig. 3). As expected, all the sprouted

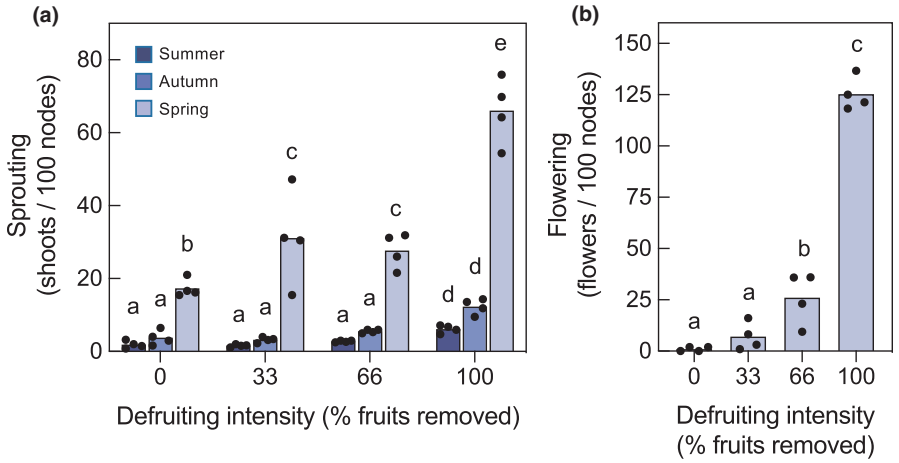

Fig. 1 Effect of fruit removal on the summer–autumn and following spring bud sprouting (a) and flowering (b) of tangor 'Nadorcott' (*Citrus clementina* × *Citrus sinensis*). Defruiting was performed in early summer. Values are the average of four trees per treatment. Different letters indicate statistically significant differences ($P < 0.01$).

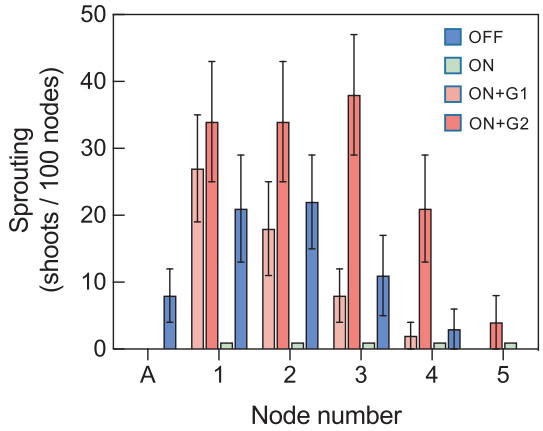

Fig. 2 Effect of fruit-peduncle girdling on axillary bud sprouting along the shoot of tangor 'Nadorcott' (*Citrus clementina* × *Citrus sinensis*). Girdling was performed on early summer (G1) and late summer (G2). Data are means ± standard error of 50 shoots per treatment distributed in 10 trees. ON: shoot with an apical fruit; OFF: shoot without fruit.

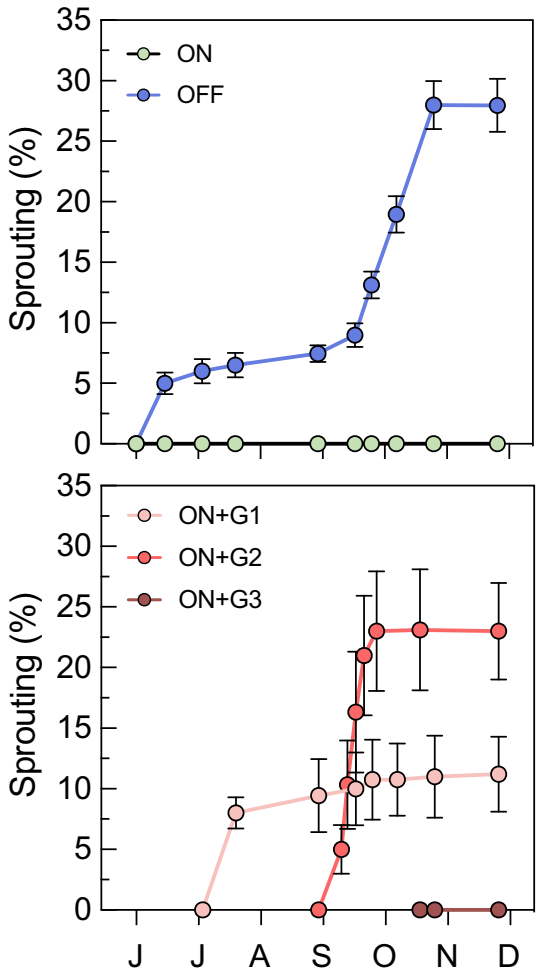

Fig. 3 Effect of fruit-peduncle girdling on the time-course of axillary bud sprouting of tangor 'Nadorcott' (*Citrus clementina* × *Citrus sinensis*) during summer and fall. Girdling was performed on early summer (G1), late summer (G2) and early fall (G3). Data are means ± standard error of 50 shoots per treatment distributed in 10 trees. ON: shoot with an apical fruit; OFF: nonfruiting shoot.

buds produced only vegetative shoots. Ferguson & Beveridge (2009) also found that, although stem-girdling blocks polar auxin transport, this not always causes bud outgrowth, because it also depends on the nutritional status of the bud (Barbier *et al.*, 2019). This, and the lower temperature for the third date of treatment, might also contribute to the lack of bud release (Fig. 3).

As a result of girdling, vegetative growth occurred precociously during late summer, and flowers were surprisingly produced in the ON-girdled shoots in the following spring, whereas the ungirdled ON shoots displayed the expected behaviour: the natural reset of vegetative growth, instead of flowering, and its transition to an OFF state (Fig. 4). The girdled shoots G1 and G2 produced 45 flowers on average, and flowers appeared only on the axillary buds formed in the new autumn shoots. The nodes from ON-girdled shoots that did not produce vegetative shoots in autumn, did not flower the following spring (Fig. 4). Accordingly, as the late-autumn girdling (ON + G3) did not induce sprouting (Fig. 3), neither did flowering (data not shown). These results suggested that flowering in the fruiting shoots was not directly due to girdling but to sprouting. Conversely, all the buds from OFF shoots had the ability to flower, even without having sprouted in autumn. In summary, these results suggested that flowering must necessarily be preceded by vegetative development. In natural conditions after an ON season, this vegetative growth generates OFF branches in spring, and the new buds flower 1 yr later, whereas girdling of ON shoots advanced vegetative growth and flowering by one season.

H3K27me3 in the *CcMADS19* locus and its repression allow flowering in the new vegetative shoots

According to the above results, girdling of fruit peduncle provides an ideal experimental set-up to address the mechanism by which the epigenetic status of *CcMADS19* is reset during vegetative growth. Our previous results comparing ON and OFF trees suggested that axillary buds were refractory to the *CcMADS19* epigenetic activation induced on neighbour leaves by the presence of the fruit. Contrary to the leaves, where the *CcMADS19* locus was enriched in the activator mark H3K4me3, this locus was enriched in the repressive mark H3K27me3 in the buds (Agustí *et al.*, 2020). Therefore, we hypothesised that new shoots and leaves generated by these buds would inherit the repressed state of *CcMADS19*.

In agreement with this hypothesis, we found that the expression of *CcMADS19* was upregulated in the leaves of ON shoots, but not in the leaves of OFF, or in the new leaves of girdled ON shoots (ON+G2) (Fig. 5a). Accordingly, with the concurrence of floral inductive signals in winter, *CiFT3* expression was maintained low in the leaves of ON shoots, compared with those of OFF or ON+G2 shoots (Fig. 5b). These results confirmed that flowering in the following spring was preceded by silencing of *CcMADS19* in ON+G2 shoots. To investigate how this silencing occurred, we examined the deposition of H3K27me3 mark in

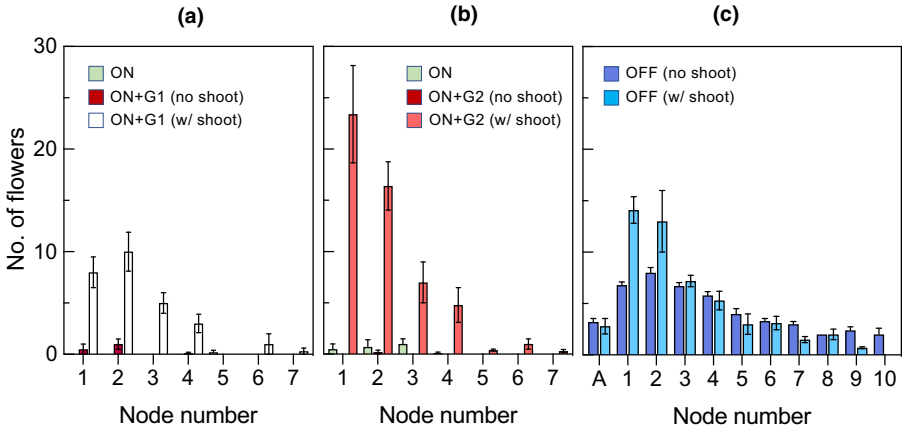

Fig. 4 Effect of fruit-peduncle girdling on summer on the flowering distribution along the ON-shoot of tangor 'Nadorcott' (*Citrus clementina* × *Citrus sinensis*) in the next spring. Girdling was performed on early summer (G1) (a) and late summer (G2) (b). OFF shoots (c) were used for comparison. Flowering was evaluated separately in shoots that had sprouted (*w/shoot*) or not (*no shoot*) in autumn. Data are means ± standard error of 50 shoots per treatment distributed in 10 trees. ON: shoot with an apical fruit; OFF: shoot without fruit.

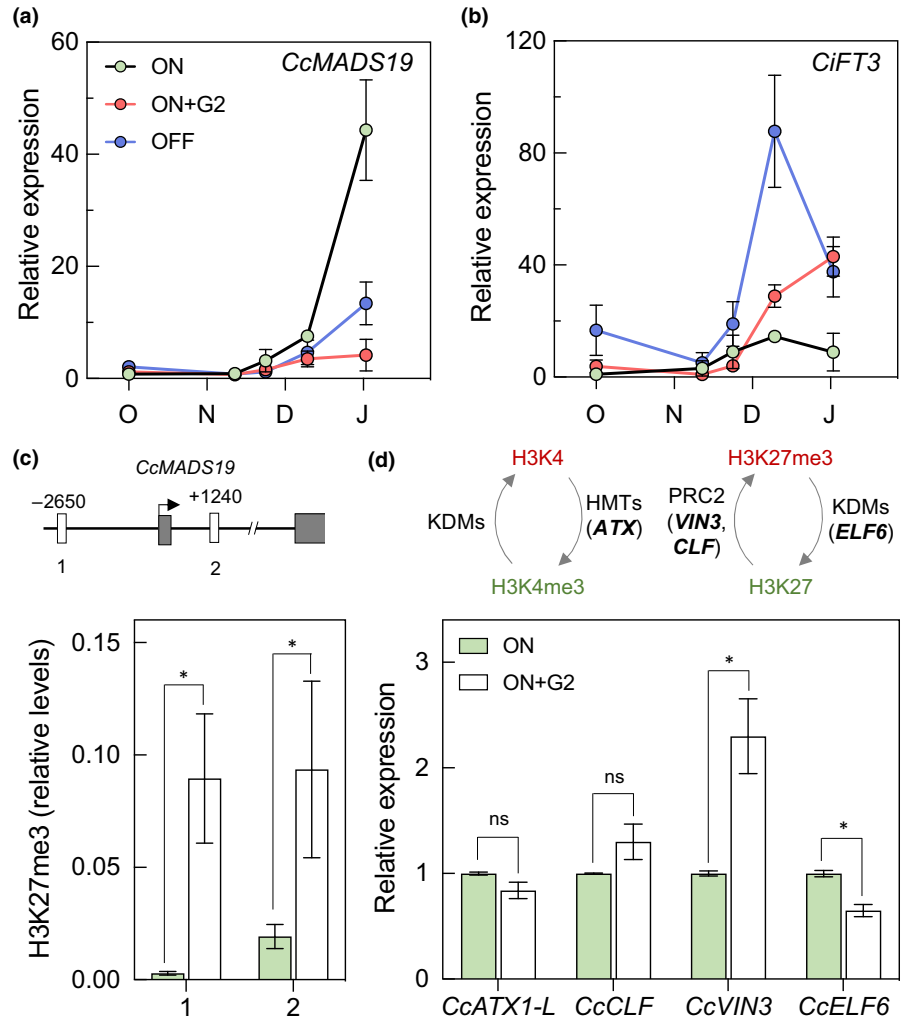

Fig. 5 *CcMADS19* active/repressed state correlates with changes in histone methylation. (a) and (b) Relative expression of *CcMADS19* and *CiFT2* in adult leaves from ON and OFF shoots, and young leaves from ON+G2 shoots. (c) H3K27me3 levels in the same leaves determined by ChIP of two regions located on the promoter of the *CcMADS-box19* locus. (d) Relative expression in adult leaves from ON shoots and young leaves from ON+G2 shoots of *CcELF6*, *CcATX1-L*, *CcCLF*, *CcVIN3* genes determined by RT-qPCR. Data correspond to leaves of tangor 'Nadorcott' (*Citrus clementina* × *Citrus sinensis*) sampled at early autumn before the inductive period (1 October). Data are means ± SE. Asterisks indicate statistical significance in Student's *t*-test ($P < 0.01$, $n = 3$); ns, not significant.

this locus, in the adult leaves of ON and the new leaves of the ON+G2 shoots. As predicted by the behaviour previously observed in buds (Agustí *et al.*, 2020), young leaves of ON+G2 shoots showed significant enrichment of the H3K27me3 in two different regions of the *CcMADS19* promoter, compared with the leaves from ON shoots (Fig. 5c).

Increase of H3K27me3 levels was further supported by the study of the expression of several *Citrus* orthologues encoding histone methyltransferases and demethylases (Figs S3, S4; Table S1). It has been reported that the PHD protein member *VIN3* and the PRC2 histone methyl-transferase *CURLY LEAF* (*CLF*) are required for the deposition of H3K27me3 marks in

the Arabidopsis *FLC* locus to repress its expression during vernalisation (De Lucía *et al.*, 2008; de Lucas *et al.*, 2016). Interestingly, we found an increase in *CcVIN3* expression in the young leaves of ON+G2 shoots, compared with those of ON shoots (Fig. 5d). This was in marked contrast with the absence of any differential effect in the expression of *CcATX1* (homologue of *TRITHORAX1*) whose *Arabidopsis* orthologue catalyses the deposition of the H3K4me3 activator mark (Pien *et al.*, 2008), or the minor reduction in *CcELF6* (*EARLY FLOWERING 6*) expression in young leaves of ON+G2 shoots (Fig. 5d). The *Arabidopsis* ELF6 protein is a H3K27me3 demethylase involved in *FLC* reactivation after vernalisation (Crevillén *et al.*, 2014). These results suggested that the maintenance of *CcMADS19* silencing in newly formed shoots and leaves could be primarily due to enhanced activity of *CcVIN3* and, consequently, the PRC2 complex and possibly to the decrease in *CcELF6* expression.

Our results are in line with the mode of flowering regulation proposed for *Arabidopsis* and *A. alpina*, in which *FLC* and *PEP1* silencing is promoted by vernalisation and mediated by H3K27me3 (De Lucía *et al.*, 2008; Wang *et al.*, 2009). Methylation begins during vernalisation in the nucleation region of the *FLC* locus and, after cold exposure, the H3K27me3 mark spreads over the entire locus to ensure maintenance of long-term silencing (De Lucía *et al.*, 2008). In *Citrus*, resetting of the competence to respond to flowering inductive signals would then be achieved by the maintenance of *CcMADS19* silencing in the buds despite their proximity to apical fruits, and subsequent vegetative growth of new shoots and leaves derived from these buds. It is noteworthy that the correlation between *CcVIN3* and H3K27me3 levels in the *CcMADS19* locus seems to occur in a context unrelated with vernalisation (i.e. October, before cold). While, in *Arabidopsis*, *VIN3* induction is triggered by exposure to low temperature (Hepworth *et al.*, 2020), and modulated by the circadian clock (Hepworth *et al.*, 2018), *CcVIN3* expression would be linked to a developmental phase transition, such as restarting vegetative growth from dormant buds. However, two alternatives are possible too. First, it cannot be ruled out that *CcVIN3* is activated by environmental signals that accompany the phase transition, which would resemble the regulation of *VIN3* in *Arabidopsis*. And second, it has been suggested that a threshold level of metabolic activity or cell division is required for vernalisation (Michaelis & Amasino, 2000); therefore, an attractive hypothesis is that sprouting could also be necessary to establish H3K27me3 marks on *CcMADS19*.

In conclusion, vegetative growth resets the fruit-dependent inhibition of flowering through epigenetic reprogramming of *CcMADS19*, which explains how the ON *Citrus* tree is able to re-establish the flowering ability during the next OFF season, therefore the new buds generated in spring on these vegetative shoots flower 1 yr later. But girdling of ON shoots advanced vegetative growth and buds flower by one season (Fig. 6). Results suggest the idea that flowering must necessarily be preceded by

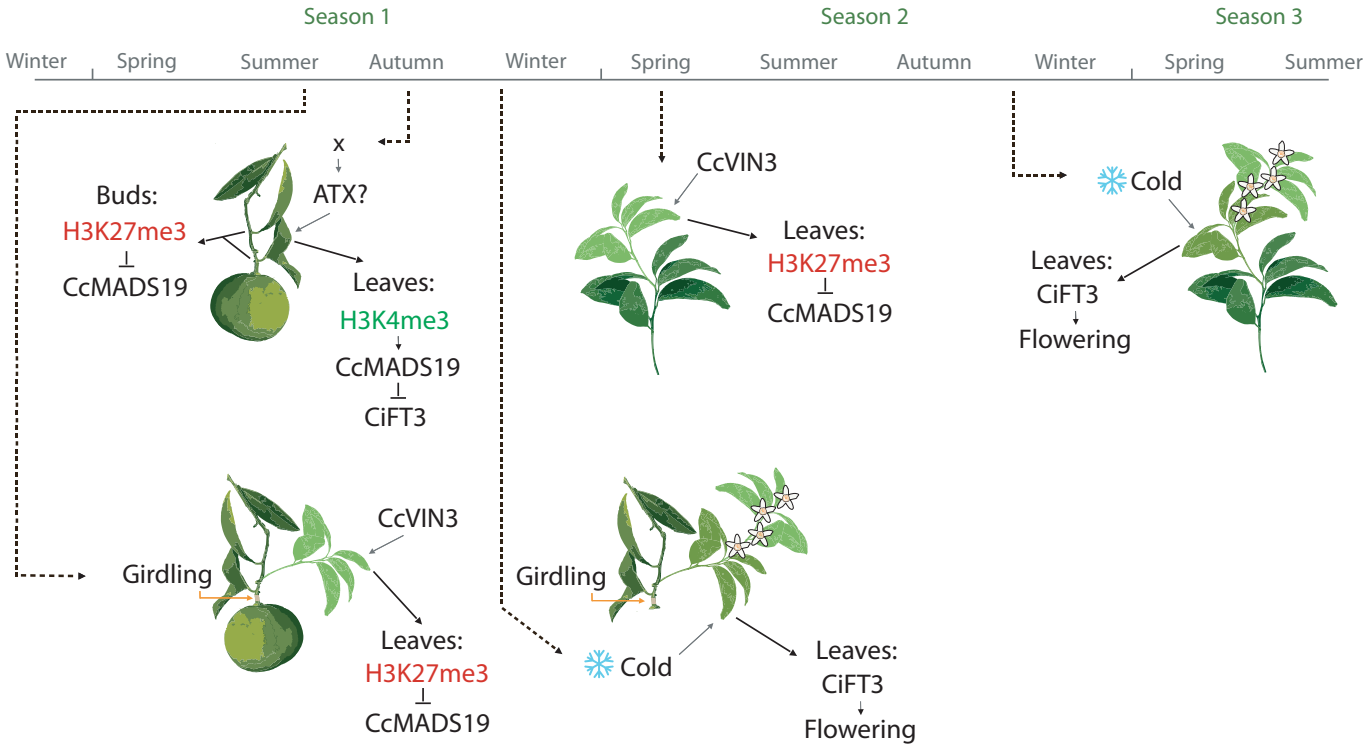

Fig. 6 Diagramatic representation of the reversion of fruit-dependent inhibition of flowering in the tangor 'Nadorcott' (*Citrus clementina* × *Citrus sinensis*). Fruit promotes H3K4 trimethylation of *CcMADS19* in nearby leaves to inhibit the response to floral inductive signals in winter, but the repressive H3K27me3 is maintained in buds. In spring, buds sprout and the newly formed leaves maintain the repressed state of *CcMADS19* thanks to enhanced *CcVIN3* activity, so that they become responsive to subsequent floral inductive cold temperatures. Summer girdling advances bud sprouting to early autumn instead of the following spring, and the new leaves are ready to respond to cold temperatures in the immediate winter, causing the induction of the florigen encoded by *CiFT3*.

vegetative development. Given that the growing fruit represses lateral bud outgrowth, the renewal of the vegetative shoots mainly occurs after the end of the ON season, with harvest, therefore giving rise to *biennial bearing*. The horticultural consequence of the latter is that promoting vegetative growth (for instance, by mechanical pruning) mitigates yield alternation in *Citrus* (Mesejo *et al.*, 2020).

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Author contributions

MA, CM, EPM and MB planned and designed the research; AM, AMF, CR and DJI performed experiments and conducted fieldwork; MdL, AMF 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.

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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 Experimental approach to force vegetative development in the axillary buds during the ON season.

Fig. S2 Effect of fruit-peduncle girdling on axillary bud IAA concentration in ON shoots with an apical fruit.

Fig. S3 Phylogenetic relationships between histone demethylase genes of *Citrus sinensis*, *Citrus clementina* and *Arabidopsis thaliana*.

Fig. S4 Phylogenetic relationships between VIN3 orthologues of several species.

Table S1 Primer sequences used in this study.

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