

Original Article

A possible role for flowering locus T-encoding genes in interpreting environmental and internal cues affecting olive (*Olea europaea* L.) flower induction

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ABSTRACT

Olive (*Olea europaea* L.) inflorescences, formed in lateral buds, flower in spring. However, there is some debate regarding time of flower induction and inflorescence initiation. Olive juvenility and seasonality of flowering were altered by overexpressing genes encoding flowering locus T (FT). *OeFT1* and *OeFT2* caused early flowering under short days when expressed in *Arabidopsis*. Expression of *OeFT1/2* in olive leaves and *OeFT2* in buds increased in winter, while initiation of inflorescences occurred in late winter. Trees exposed to an artificial warm winter expressed low levels of *OeFT1/2* in leaves and did not flower. Olive flower induction thus seems to be mediated by an increase in FT levels in response to cold winters. Olive flowering is dependent on additional internal factors. It was severely reduced in trees that carried a heavy fruit load the previous season (harvested in November) and in trees without fruit to which cold temperatures were artificially applied in summer. Expression analysis suggested that these internal factors work either by reducing the increase in *OeFT1/2* expression or through putative flowering repressors such as TFL1. With expected warmer winters, future consumption of olive oil, as part of a healthy Mediterranean diet, should benefit from better understanding these factors.

Key-words: alternate bearing; flowering; flowering locus T; fruit load; *Olea europaea*; olive.

INTRODUCTION

The cultivated olive (*Olea europaea* ssp. *europaea* var. *europaea*) was likely domesticated ~6000 years ago in the northeast Levant (current border between Turkey and Syria), and perhaps co-domesticated in additional locations in the Mediterranean basin (Zohary and Spiegel-Roy 1975; Besnard *et al.* 2013; Diez *et al.* 2015). The evergreen olive tree's

productivity and survival are threatened by temperatures below -7 and -12°C , respectively (Pallioti and Bongi 1996; Barranco *et al.* 2005). Nevertheless, cold winter temperatures are required for olive flowering (Hartmann and Porlingis 1953). Potted olive trees from several cultivars subjected to winters with a minimum temperature of 15.5°C in a greenhouse did not flower (Hartmann and Porlingis 1957). In the Mediterranean basin, December and January are relatively cold months, and the Mediterranean climate is thus suitable for both olive tree survival and productivity. The olive is deeply embedded in Mediterranean culture and in human history (Loumou and Giourga 2003). Current global annual production of olive oil is above three million tons (International Olive Council 2015), mostly in the Mediterranean basin but also in large quantities in additional Mediterranean climates within Argentina, Australia, China and the USA.

Olive cultivars are vegetatively propagated clones, with a high level of heterozygosity because olives are self-incompatible (Breton *et al.* 2014) and wind pollinated (Cuevas and Polito 2004). When grown from seed, the olive tree will likely have a juvenile phase of over 10 years. In one case study, only 21% of olive seedlings reached a complete adult stage after 12 years (Bellini 1992). When the mature phase is reached, in some of the lateral meristems on 1-year-old shoots, the first clear anatomical change in the bud, indicating inflorescence initiation, is obvious towards the end of winter (Hartmann 1951; Fabbri and Alerci 1999). The apical bud usually remains vegetative. The percentage of lateral buds that form inflorescences is highly variable (0 to 95%) (Lavee 1996). In spring, many lateral buds are 'released', resulting in outgrowth of an inflorescence or a vegetative shoot. An olive inflorescence reaches anthesis in the northern hemisphere between April and June, depending on genotype and spring temperatures (Osborne *et al.* 2000; El Yaacoubi *et al.* 2014). An inflorescence typically consists of 10–32 male or hermaphrodite flowers, and on average, 0.1–0.7 (depending on genotype) fruits per inflorescence survive till ripening (Lavee 2007). Mature fruits are normally harvested in the northern hemisphere during October–December (Dag *et al.* 2011; Camposeo *et al.* 2013).

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A major factor affecting final olive yield is the number of inflorescences reaching anthesis in the spring (Lavee 2007). This number mostly depends on the number of new lateral buds on the tree and the percentage of these buds that form inflorescences. Heavy fruit load (HFL) dramatically reduces olive shoot growth, thus limiting the number of new lateral meristems that can potentially form inflorescences (Dag *et al.* 2010; Smith and Samach 2013). Although not yet tested in olive, in other fruit trees, there is growing evidence that HFL directly inhibits flower induction (Nakagawa *et al.* 2012; Nishikawa *et al.* 2012; Samach and Smith 2013; Ziv *et al.* 2014; Haberman *et al.* 2016). In any case, in olive, a branch with HFL will not initiate inflorescences the following winter and will thus carry no fruit the following summer.

Girdling a single branch/scaffold within an olive tree has little effect on flowering of the rest of the tree (Lavee *et al.* 1983), suggesting that branch autonomy (Sprugel *et al.* 1991) in olive is quite pronounced. Still, some neighbouring adult trees of the same olive cultivar within an orchard have HFL on all branches (designated as 'on' year), while others have almost no fruit at all ('off' year). Thus, the autonomous branches within a tree appear to be highly synchronized. Moreover, in many cases, almost all trees within an olive orchard and sometimes even within a growing region are synchronized in either an 'on' or 'off' year (Lavee 2007). This leads to a biennial cycle of fruiting in the orchard, also termed 'alternate bearing'. In olive, the difference in yield between 'on' and 'off' years may reach 20 t ha⁻¹ (Lavee 2007). A similar, albeit mostly less extreme fruiting pattern is common to many species of fruit trees (Smith and Samach 2013). Clearly, this is detrimental for commercial cultivation of fruit crops (Jonkers 1979), and a reduction in its amplitude normally requires farmer intervention, such as flower/fruitlet thinning at the beginning of an 'on' year (Dennis 2000). To simplify, we use the term 'year' to describe the period between one anthesis and the next (late spring). Thus, even though olives are harvested in the autumn, an 'on' year lasts, based on this definition, until the next spring, when the level of anthesis will likely be very low or non-existent, beginning a 12 month 'off' year. During winter, none of the trees have flowers or fruit, yet we term them 'on' or 'off' based on their fruit load during the previous summer.

Several experiments repeated with variations over the years have shown that complete olive fruitlet removal up to a certain date in early summer can still eliminate or reduce the HFL-dependent reduction in the next year's olive flowering. After this date (which changes with the experiment), towards the end of summer and perhaps in correlation with endocarp sclerification (formation of the olive pit surrounding the embryo), the reduction in flowering is irreversible (Fernandez-Escobar *et al.* 1992; Dag *et al.* 2010). In 1990, two groups reported histochemical changes as well as changes in overall RNA levels in meristems during the summer (Navarro *et al.* 1990; Pinney and Polito 1990). Although, as far as we know, the actual data were not published, these reports, together with the above-mentioned fruit removal studies, as well as findings that gibberellin injections during the summer

can inhibit flowering (Fernandez-Escobar *et al.* 1992), led to the assumption that flower induction in olive occurs before winter, around the time of endocarp sclerification (Fernandez-Escobar *et al.* 1992). For an explanation of the findings from the elegant experiments performed by Hartmann and his colleagues in which cold winters were essential for olive flower initiation (Hartmann and Porlingis 1957), it was proposed that winter chilling releases pre-formed olive floral buds from dormancy (Rallo and Martin 1991; Rallo *et al.* 1994). To the best of our knowledge, this remains the consensus regarding olive flowering (Ulger *et al.* 2004).

Since the 1990s, great progress has been made in identifying proteins that either induce or inhibit flower induction in plants (Andrés and Coupland 2012). For example, once a plant apical or lateral meristem accumulates high levels of the flowering locus T (FT) protein, initially identified in *Arabidopsis thaliana* (Kardailsky *et al.* 1999; Kobayashi *et al.* 1999), it will likely transform into an inflorescence meristem. In many cases, following the pattern of accumulation (inducers) or reduction (inhibitors) of these proteins' transcripts clearly reveals the time of flower induction for each species. FT, for example, can travel in the phloem to the meristem, and transcript accumulation may therefore occur in the leaves (Corbesier *et al.* 2007; Tamaki *et al.* 2007). The accumulation of FT-like gene transcripts has been documented as preceding flower initiation in several fruit trees, such as citrus (Nishikawa *et al.* 2007; Muñoz-Fambuena *et al.* 2011; Shalom *et al.* 2012), mango (*Mangifera indica* L.) (Nakagawa *et al.* 2012), avocado (*Persea americana* Mill.) (Ziv *et al.* 2014) and apple (*Malus domestica* Borkh.) (Kotoda *et al.* 2010). In several species, HFL inhibits FT accumulation (Muñoz-Fambuena *et al.* 2011; Nakagawa *et al.* 2012; Ziv *et al.* 2014).

On the other hand, accumulation of the flowering inhibitor terminal flower 1 (TFL1) in *Arabidopsis* meristems can delay flowering (Shannon and Meeks-Wagner 1991; Bradley *et al.* 1997). TFL1, although similar in structure to FT, seems to be antagonistic to the latter. The interaction between these two proteins, each encoded by a small gene family in every species, determines flower induction, indeterminacy of the inflorescence and additional plant architectural traits (Shalit *et al.* 2009). This activity of TFL1-like proteins has been demonstrated in various other plant species (Pnueli *et al.* 1998; Mimida *et al.* 2009; Mohamed *et al.* 2010; Freiman *et al.* 2012; Iwata *et al.* 2012; Randoux *et al.* 2012). Transcripts of a TFL1-encoding gene were seen to accumulate in apple meristems in response to HFL (Haberman *et al.* 2016).

Here we first attempted to clarify, using histology and molecular markers, when the inflorescence meristems are formed in olive. We then sought to identify and follow FT-encoding genes to better determine when flower induction occurs. We further investigated how HFL affects flower induction and clarified the role of cold winter temperatures in olive flowering, either through induction or by releasing pre-formed inflorescences from dormancy. We show that FT overexpression causes early flowering in olive and that olive FT-encoding genes cause early flowering when ectopically expressed in *Arabidopsis*. Our results suggest that flowering induction, as reflected by FT accumulation, occurs during the winter in response to cold

temperatures. Exposing olives to cold temperatures induces FT expression and can cause out-of-season flowering. The 'biochemical memory' of fruit load has an inhibitory effect on FT accumulation, and perhaps on its function, by increasing flowering inhibitors such as TFL1.

MATERIALS AND METHODS

Plant material

Olive

Olive cv. Barnea (Lavee *et al.* 1986), used in all of the experiments, is propagated by rooting of vegetative cuttings, with no use of rootstocks. The rooted branch usually develops in the nursery for 1–2 years before being planted in an orchard.

Orchard experiments were performed in an irrigated, commercially cultivated orchard ('Gadash Tsabar Kama') located in the southern coastal plain of Israel (31°44'52.95"N, 34°51'08.87"E), planted with trees in 2001. The experiments were conducted during the seasons 2009–2010, 2010–2011 and 2013–2014. Thus, the youngest trees were ~10 years old and the oldest were ~14 years old (Supporting Information Fig. S1d). Trees in controlled-environment experiments (seasons 2014–2015 and 2015–2016) were rooted in 2010 (~4–6 years old) and grown in 25 L pots (Supporting Information Fig. S1a–c).

Embryogenic cultures derived from a mature embryo of the olive cv. Picual were used for transformation experiments.

Arabidopsis

Arabidopsis transgenic *FILpro:LHG4* plants on the Landsberg erecta (Ler) background (Goldshmidt *et al.* 2008) were used for functional analysis of *OeFT1* and *OeFT2* cDNA using a trans-activation system (Moore *et al.* 1998), forming transgenic lines *FILpro>>OeFT1* and *FILpro>>OeFT2*.

Gene cloning and constructs

See the Materials and Methods section in the supporting information.

Plant transformation and selection of transgenic plants

See the Materials and Methods section in the supporting information.

Olive tree treatments

Choosing trees with different fruit loads. In the summer of 2009, five uniform trees that flowered extensively and carried a high number of fruitlets were selected and designated 'on' trees while five additional trees, in the same block, with very few fruitlets, were selected and designated as 'off' trees. See Supporting Information Fig. S1d for more information on estimated fruit numbers. In the following years, the same 10 trees

were used in the experiments. However, because of the alternating-year switch in the following year, trees that were in an 'on' year entered in an 'off' year.

Fruit removal. For the fruit removal treatments, trees in an 'on' year were selected in the summers of 2009 and 2015. In each tree, two limbs with a main branch (diameter of 10–14 cm) were chosen for fruit removal and control. These limbs constituted roughly 16% of the tree. On the date of fruit removal (8 Jul or 18 Aug 2009 and 28 May 2015), all fruitlets were removed from one of the tree limbs (in five trees per removal date), leaving the second limb as an internal control (HFL).

Controlled environment. The controlled-environment experiments were conducted at the Robert H. Smith Faculty of Agriculture, Food and Environment, Rehovot, Israel, in two facilities: a heated glasshouse where temperature was kept above 15 °C at all times and ranged between 15 and 30 °C, under natural light and day length, and 'phytotron' rooms with different temperature regimes (28/22 and 16/10 °C day/night temperatures). Changes between day and night temperatures were gradual, spanning 3 h (Sobol *et al.* 2013). Potted trees transferred to the phytotron rooms on 25 Nov 2014 were exposed to short-day lighting conditions (9/15 h light/dark cycles), maintained by transferring pots daily into dark rooms with appropriate temperatures. Potted trees transferred to the phytotron rooms on 22 Jun and 1 Sep 2015 were exposed to long days (16/8 h light/dark cycles), maintained by extending the natural day length with additional lighting (3–5 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation), using 75 W incandescent tungsten bulbs (LM960 Osram GmbH, Munich, Germany). The glass-covered growth rooms transmitted ~80% of outside solar radiation.

The number of hours below 15 °C was calculated from hourly ambient temperature measurements, from a nearby meteorological station (Rehovot station) of the Israeli Ministry of Agriculture and Rural Development, obtained through the website www.meteo.co.il

Flowering measurements

Olive

Rate of flowering was determined either by visual assessment in the following spring, whereby each tree or tree limb was given a flowering score on a scale of 0–5, '5' representing the highest flowering intensity, or according to flowering in pre-selected shoots. In each shoot, the six buds in the three nodes described for the tissue sampling (see further on) were surveyed for the presence of inflorescences. Average percent flowering for the shoots was calculated as representing tree flowering.

Arabidopsis

T2 generation transgenic *FILpro>>OeFT1* (four lines) or *FILpro>>OeFT2* (two lines) from each of the lines were grown in growth chambers under short-day conditions (23/21 °C day/night, 10/14 h light/dark), along with *FIL:LHG4* plants as a control. Flowering time was determined by counting the

number of rosette and cauline leaves until the first flower bud. Each of the T2 lines segregated for the transgene. Thus, some of the progeny were not transgenic and flowered late, similar to the *FIL:LHG4* plants. For each line, we measured flowering of only the transgenic progeny, 7–18 plants per line.

Scanning electron microscopy

Samples were fixed in 0.1 M phosphate buffer (pH 7.2) containing 5% (w/v) glutaraldehyde for 24 h, followed by five washes in phosphate buffer. The tissues were gradually dehydrated with increasing concentrations of ethanol. Tissues were then dried in a critical point dryer device (BAL-TEC CPD-030, Bal-Tec AG, Balzers, Liechtenstein). Bud leaf primordia were removed under a binocular microscope, revealing the meristem. Dissected buds were sputter coated with gold [Polaron scanning electron microscope (SEM) coating unit, Polaron Instruments, Hatfield, PA, USA]. Images of the meristems were produced in an SEM (Jeol SEM, JSM-5410 LV, Tokyo, Japan).

Gene expression

Tissue collection, transgenic *Arabidopsis*. For the determination of expression levels of the transgene, T3 plants, descendants of a specific early flowering T2 plant, were sown, and after 15 d, the above-ground part of the seedling was collected. Each sample was composed of 10 seedlings. RNA was extracted from the tissues.

Tissue collection, transgenic olives. Total RNA was extracted from control and transgenic olive embryogenic calluses at the same stage (4 weeks after subculture).

Tissue collection, olive experiments. From each tree at every time point, four shoots that developed in the concurrent season were sampled. From each sampled shoot, the first node (first from the opposite end of the apical bud) was removed, and the segment with the following four nodes was collected (see Supporting Information Fig. S2). The shoot segment was separated into different tissues (leaf, petiole, stem and bud). In some samples, the stem and bud tissues were processed together. Tissues from every four shoots from the same tree were combined to represent that tree. Sampling time during the day was around 5 h after sunrise. Samples for RNA extraction were placed in liquid nitrogen immediately after sampling and stored in a -80°C freezer.

RNA extraction and first-strand cDNA synthesis. See the Materials and Methods section in the supporting information.

Expression analysis by quantitative real-time PCR. See the Materials and Methods section in the supporting information.

Statistical analysis. Data from quantitative real-time PCR (qPCR) results and flowering were analysed by one-way analysis of variance (ANOVA) using JMP version 10 software (SAS Institute, Cary, NC, USA). Differences between treatments were determined by Student's *t* test. In multiple comparisons, Tukey–Kramer honestly significant difference (HSD) was implemented. Statistical significance was determined at $P \leq 0.05$. In cases where the variance was unequal and the data did not show a normal distribution, the statistical tests were conducted on transformation to ranks of the values.

RESULTS

Timing of olive inflorescence initiation

In the summer of 2009, we selected, followed and sampled neighbouring 'on' and 'off' 'Barnea' olive trees in a commercial orchard. As expected, 'off' trees flowered extensively the following spring (Fig. 1e). We specifically followed the fate of certain lateral buds in these 'off' trees (Materials and Methods section, Fig. 1d and Supporting Information Fig. S2 for position of these buds) by SEM. These buds initially contained a vegetative meristem, forming leaf primordia (Fig. 2a–f). The first noticeable change occurred in February, when no new leaf primordia were formed and the meristem seemed to bulge, forming a dome shape (Fig. 2g,h). Once the bud apex became floral, the most recently formed leaf primordia developed into bracts subtending inflorescence meristems (Fig. 2j,k). In contrast, adjacent 'on' trees of the same cultivar, selected based on HFL in summer, initiated relatively few inflorescences the following spring (Fig. 1e). The meristems in lateral buds of 'on' trees did not appear to go through a flowering transition, remaining vegetative into February (Fig. 2l).

In *Arabidopsis*, flower primordia initiating from the inflorescence meristem accumulate transcripts of the meristem identity transcription factor *APETALA1* (*AP1*) (Mandel *et al.* 1992). Later, the central region of a flower primordium accumulates transcripts encoding the conserved organ identity MADS box protein *AGAMOUS* (*AG*) (Yanofsky *et al.* 1990). Olive transcripts encoding similar proteins (*OeAPI-1* and *OeAG-1*) were cloned (Supporting Information Data S1), and expression of these genes was measured in lateral buds (position of buds similar to those chosen for SEM analysis) during the fall and winter of 2009–2010 and in developing inflorescences (February 2010). Expression of both genes was highest in inflorescences (Fig. 3). A significant increase in *OeAPI-1* expression in buds from 'off' trees was detected in January 2014, before inflorescence initiation (Fig. 3c). Thus, microscopy and molecular markers suggested that under local conditions, 'Barnea' initiates inflorescence towards the end of winter (January to February), and this occurs in trees in which previous crop load was low.

High levels of FT cause precocious flowering in transgenic olives

In several plant species, a seasonal increase in FT-encoding transcripts is associated with flower induction, an event preceding inflorescence initiation (Andrés and Coupland 2012). The *MtFta1* gene controls flowering time in *Medicago truncatula* (Laurie *et al.* 2011). A mutation in the gene causes late flowering, and the gene encodes an FT-like protein that, when overexpressed, can cause early flowering in *M. truncatula* and in *Arabidopsis* (Laurie *et al.* 2011). The olive juvenile period can be shortened by as much as 28 months using certain cultural practices (Santos-Antunes *et al.* 2005). Based on a relatively new olive transformation protocol (Torreblanca *et al.* 2010), we produced transgenic olive plants overexpressing the

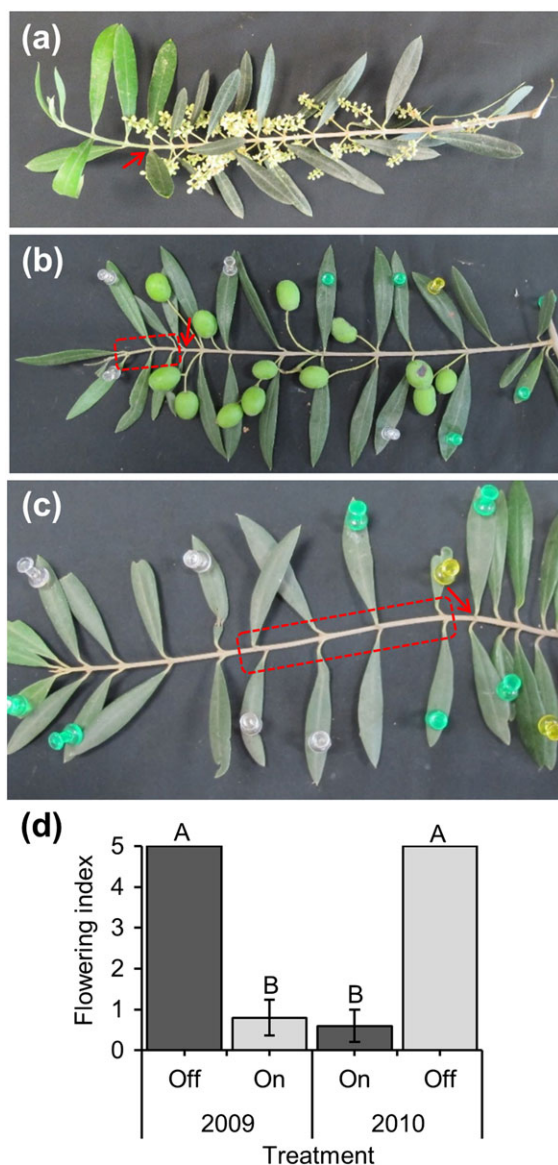


Figure 1. Flowering and growth in response to fruit load. (a–c) Pictures of olive shoots. (a) A shoot at anthesis from a tree entering an ‘on’ year. (b) A shoot bearing fruit in late August, from an ‘on’ tree. (c) Shoots bearing no fruit in late August from an ‘off’ tree. The red arrow in a–c marks the postulated first node of the new vegetative growth in spring. All shoots produced new leaf primordia, but more were formed on branches with no fruit. In b and c, the shoot segment sampled and analysed for gene expression, scanning electron microscope (SEM) imaging and flowering is indicated by a red dashed square frame. (d) ‘On’ and ‘off’-year trees were selected in the summer of 2009 according to their degree of fruit load. Average score of flowering intensity per tree was estimated the following spring during two seasons (2010 and 2011). The light grey columns represent trees that were ‘on’ in 2009 and therefore ‘off’ in 2010. The dark grey columns represent trees that were ‘off’ in 2009 and therefore ‘on’ in 2010. Every spring, each tree was given a flowering score on a scale of 0–5, ‘5’ representing the highest flowering intensity. Numbers are mean values of five biological repeats (trees) $\pm$ standard error of the mean (SE; bars). Bars for ‘off’-year trees are not visible owing to low standard error between trees. Different letters indicate a significant difference between treatments according to Tukey–Kramer honestly significant difference (HSD) test on ranked data ($P \leq 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com]

MtFTa1 gene. Sixteen independent transgenic lines were recovered, yielding a 2.56% transformation rate. Fifteen lines formed mature embryos that germinated. *MtFTa1* gene expression was detected in calli of nine transgenic lines (Supporting Information Fig. S3). Control non-flowering micro-propagated shoots showed monopodial branching associated with marked apical dominance producing a small number of axillary shoots (Fig. 4a,e and Supporting Information Table S1). Early flowering in three transgenic lines was observed *in vitro* about 2–20 weeks after somatic embryo germination (Fig. 4b and Supporting Information Fig. S4). Single flowers formed in apical meristems, leading to abnormal (for olive) sympodial growth. The solitary flower reached anthesis after 12–14 d from when it first appeared. The repetitive conversion of the apical meristems to floral buds causes continuous growth of lateral shoots (Fig. 4b–d and Supporting Information Fig. S4, Table S1). Micro-propagation of flowered *MtFTa1* shoots was difficult because most vegetative buds had given rise to floral buds. Several plants from the early flowering transgenic lines were acclimated to *ex vitro* conditions in a growth chamber. Plants of the early flowering FT7 line were successfully maintained in a confined greenhouse (Fig. 4c,d). These plants produced new flowers all year-round, although flowering appeared to be more pronounced at the end of winter. They have set fruit after being fertilized with pollen from cv. Arbequina.

FT-encoding genes from olive promote early flowering in transgenic *Arabidopsis*

We cloned two olive genes from ‘Barnea’, *OeFT1* and *OeFT2*, encoding proteins similar to FT (Supporting Information Data S1 and Fig. S5). Based on recently published olive genome data (Cruz *et al.* 2016), there are no additional genes encoding FT. We formed transgenic *Arabidopsis* plants harbouring two constructs based on a trans-activation system (Moore *et al.* 1998): a driver construct expressing the synthetic transcription factor LHG4 under the *filamentous flower* (*FIL*) promoter (*FILpro*: *LHG4*) and a responder construct expressing our genes of interest (*OeFT1* or *OeFT2*) downstream of five or six copies of the *Escherichia coli* operator, recognized by LHG4 (5XOP: *OeFT1* or 6XOP: *OeFT2*). Plants that harbour both constructs (*FILpro* $\gg$ *OeFT1* or *FILpro* $\gg$ *OeFT2*) express the gene of interest in the expression domain of the specific promoter (Supporting Information Fig. S6b). The *FIL* promoter is active primarily in leaf primordia and not in meristems (Lifschitz *et al.* 2006).

T2 generation *FILpro* $\gg$ *OeFT1* and *FILpro* $\gg$ *OeFT2* seedlings were grown under short-day conditions (10 h light/14 h dark), which delay flowering in *Arabidopsis*, and the final leaf number was determined and compared to control plants (*FILpro* without responder construct) grown under the same conditions. The transgenic plants flowered significantly earlier than the control plants (Fig. 4f,g): while the latter flowered after ~29 leaves, the transgenic *OeFT2* lines flowered with ~15 leaves and the transgenic *OeFT1* lines with four to nine leaves

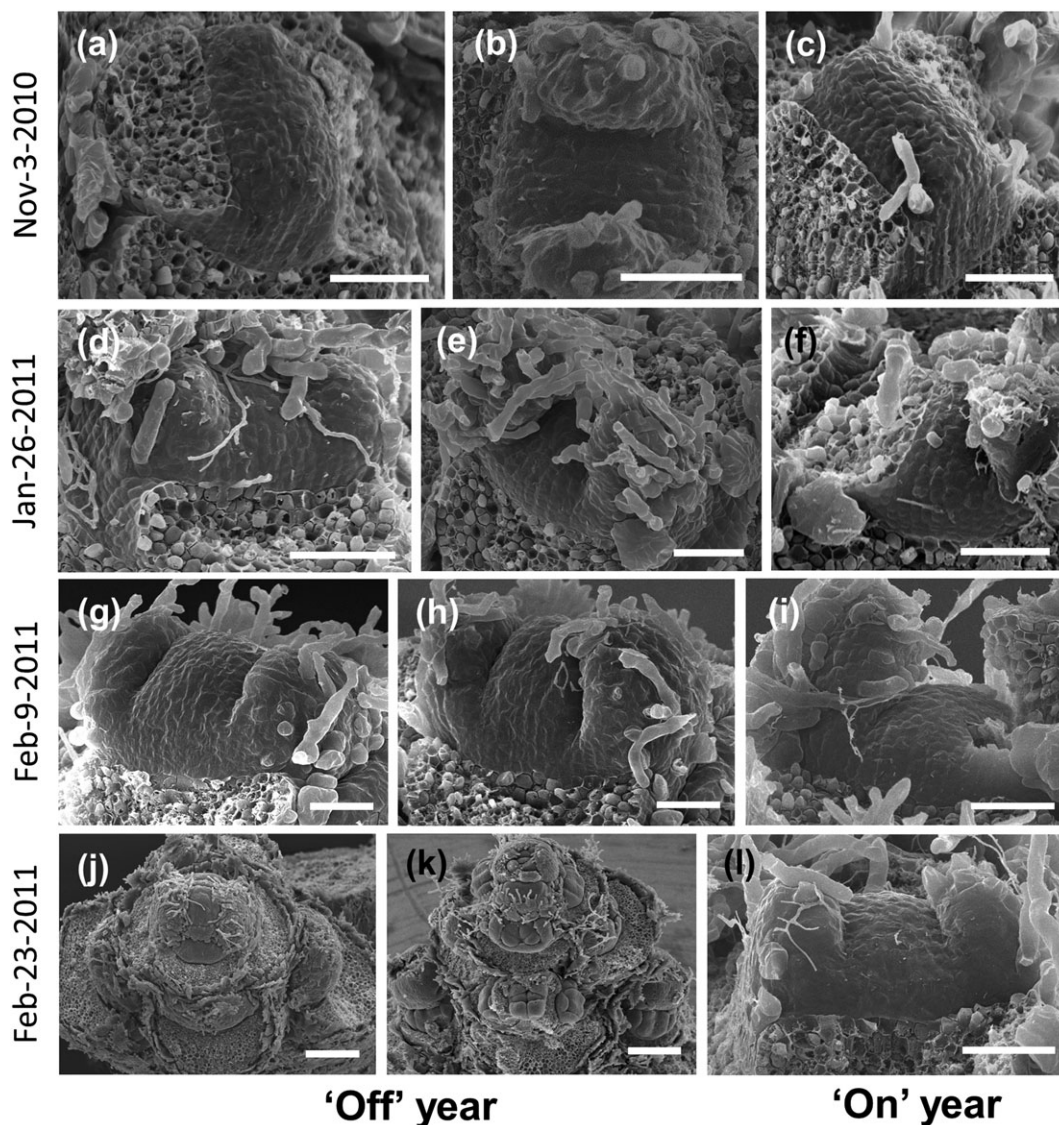


Figure 2. Inflorescence formation. Scanning electron microscope (SEM) images of the meristem in lateral buds before and during the transition to flowering. Buds were collected from 'off'-year (left and middle columns; a, b, d, e, g, h, j, k) or 'on'-year trees (right column; c, f, i, l). Buds were collected on 3 Nov 2010 (first row; a–c), 26 Jan 2011 (second row; d–f), 9 Feb 2011 (third row; g–i) and 23 Feb 2011 (fourth row; j–l). Before February, we could not distinguish differences between buds from 'off' and 'on' trees, with meristems forming leaf primordia. On 23 Feb, meristems in buds from 'off'-year trees are forming inflorescences (j, k), while buds from 'on'-year trees keep forming leaf primordia (l). Each image represents several samples examined for the specific treatment. Scale bar is 0.05 mm (a–i, l) or 0.25 mm (j–k).

(Fig. 4g). Earlier flowering using *OeFT1* compared to *OeFT2* cDNA did not correlate with higher expression of the former construct (Supporting Information Fig S6b; see discussion for other possible explanations).

Expression of FT-encoding genes prior to inflorescence initiation

Relative expression levels of *OeFT1* and *OeFT2* in the different tissues were determined before flower initiation (19 Jan 2014) in trees in both 'off' and 'on' years. By sampling the shoot segment at the base of the flush (Materials and Methods section), we compared leaves and buds of a similar developmental

state in 'on' and 'off' trees. Compared to gene expression in leaves and lateral buds, expression of both genes in petioles and stems was very low and did not differ between trees with different fruit loads. *For both genes, the highest relative expression was measured in leaves from 'off' trees (Fig. 5a,b), while expression in 'on' tree leaves was significantly lower. Expression in lateral buds was only detected for the *OeFT2* gene and specifically in 'off' trees (Fig. 5b).

We then followed seasonal changes in the expressions of *OeFT1* and *OeFT2* in leaves of the same tree during two consecutive seasons (2009–2011). We compared trees that began as 'on' and proceeded to 'off' the following year and those that began as 'off' and proceeded to 'on' the following year. For both genes, in both years, in all trees, the expression was

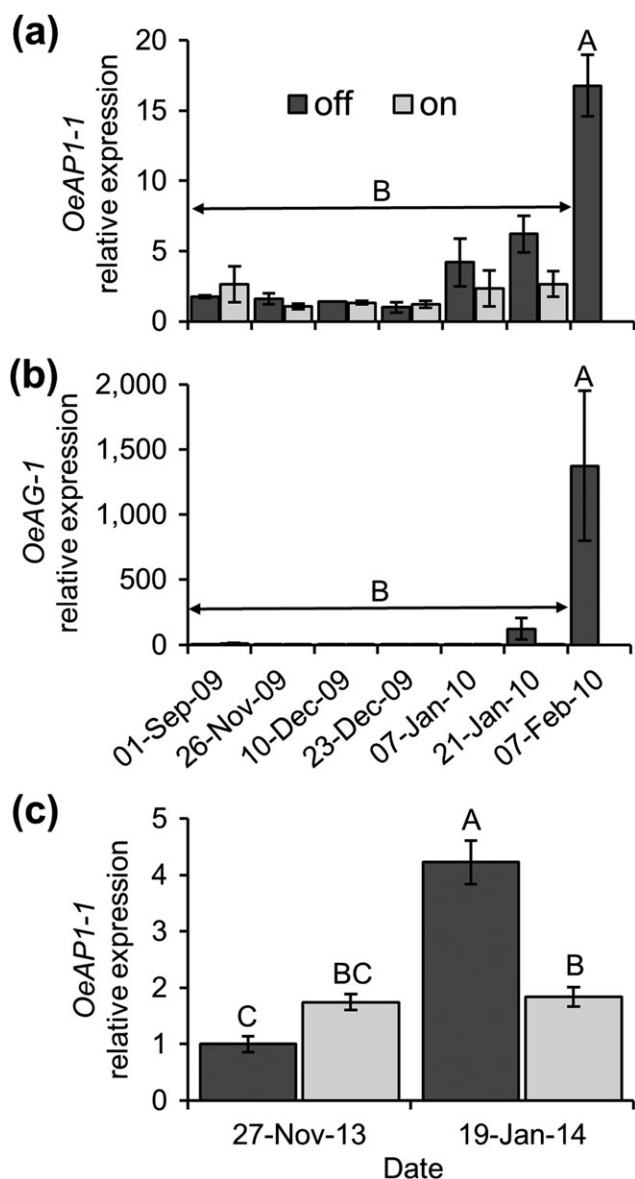


Figure 3. Timing the transition to flowering with molecular markers. Relative expression of putative meristem and organ identity genes in lateral buds from 'on' and 'off' trees. (a, b) Stems with lateral bud samples, collected during the winter of 2009–2010. In samples collected from 'off'-year trees on 7 Feb 2010, inflorescences had initiated from the buds. (c) Buds collected during 2013–2014. Relative expression of *OeAPI-1* (a, c) and *OeAG-1* (b) was measured using quantitative real-time RT-PCR (qPCR; Materials and Methods section). Numbers are mean values of three (a, b) or five (c) independent biological repeats (trees) $\pm$ SE (bars). Different letters represent significant differences between treatments according to Tukey–Kramer honestly significant difference (HSD) test ($P \leq 0.05$).

baseline during the summer and before December and then started to increase from December, peaking at the end of January (Fig. 5c,d), before inflorescence initiation. Levels were consistently higher in leaves from trees that were concurrently in their 'off' year (Fig. 5a–d). Once again, *OeFT2* accumulation in lateral buds occurred in January, specifically in concurrently 'off' trees (Fig. 5e,f).

Thus, in 'Barnea' trees under local conditions, *OeFT1/2* expression in leaves began to increase in December–January, inflorescence initiation occurred in February and trees reached anthesis in March. Towards the end of winter, 'on'-year trees had significantly lower *OeFT1/2* expression in leaves and no detectable expression of *OeFT2* in lateral buds. The rise in *OeFT1/2* expression therefore seems to correlate well with flower induction. Olives normally ripen after the summer and are harvested in the autumn. Thus, fruits are no longer present when *OeFT1/2* gene expression increases. Nevertheless, the rate of accumulation of these transcripts is affected by the 'biochemical memory' of previous fruit load on the tree.

Effect of early fruit removal on FT transcript accumulation and subsequent flowering

In a previous study, removing fruit as early as mid-October was not sufficient to erase the 'biochemical memory' of fruit load in the olive cv. Coratina, but earlier removal of fruit (mid-August or earlier) significantly improved flowering the following spring (Dag *et al.* 2010). We asked if early fruit removal would affect *OeFT1/2* accumulation during the winter and if this change in expression would correlate with return flowering. In the summer of 2009, complete fruit removal was performed on a major branch of 'on' 'Barnea' trees, leaving a similar branch on the same tree with its fruit untouched until harvest (internal control). When we compare fruit removal on 8 Jul with fruit removal on 18 Aug, the earlier fruit removal allowed significantly higher accumulation of *OeFT1/2* in leaves during December–January (Fig. 6c,e). The earlier fruit removal also led to a significantly higher rate of flowering in the spring (Fig. 6a). The number of nodes formed by shoots was significantly increased by early fruit removal, in comparison to shoots of 'on' trees (Supporting Information Fig. S7).

In a similar experiment in the summer of 2015, complete fruit removal from a single major branch of an 'on' tree on 28 May allowed ~22% flowering on this branch while the neighbouring branches carrying fruit till harvest did not flower at all (Fig. 6b). Expression of *OeFT1* in leaves on 4 Jan 2016 was significantly higher in branches from which fruit had been removed on 28 May 2015 (Fig. 6d). Here, the increase in expression of *OeFT2* due to early fruit removal was not significant (Fig. 6f). These experiments suggest that fruit removal up to a certain date leads to a diminished 'biochemical memory' of fruit load during winter flower induction.

Shortening the natural winter by exposing trees to warmer conditions can affect expression of FT-encoding genes and return flowering

December and January are months of relatively cold temperatures in the Mediterranean, and cold temperatures have been shown to promote olive flower initiation (Hackett and Hartmann 1967). We conducted experiments similar to those performed by Hartman and colleagues ~50 years ago to study

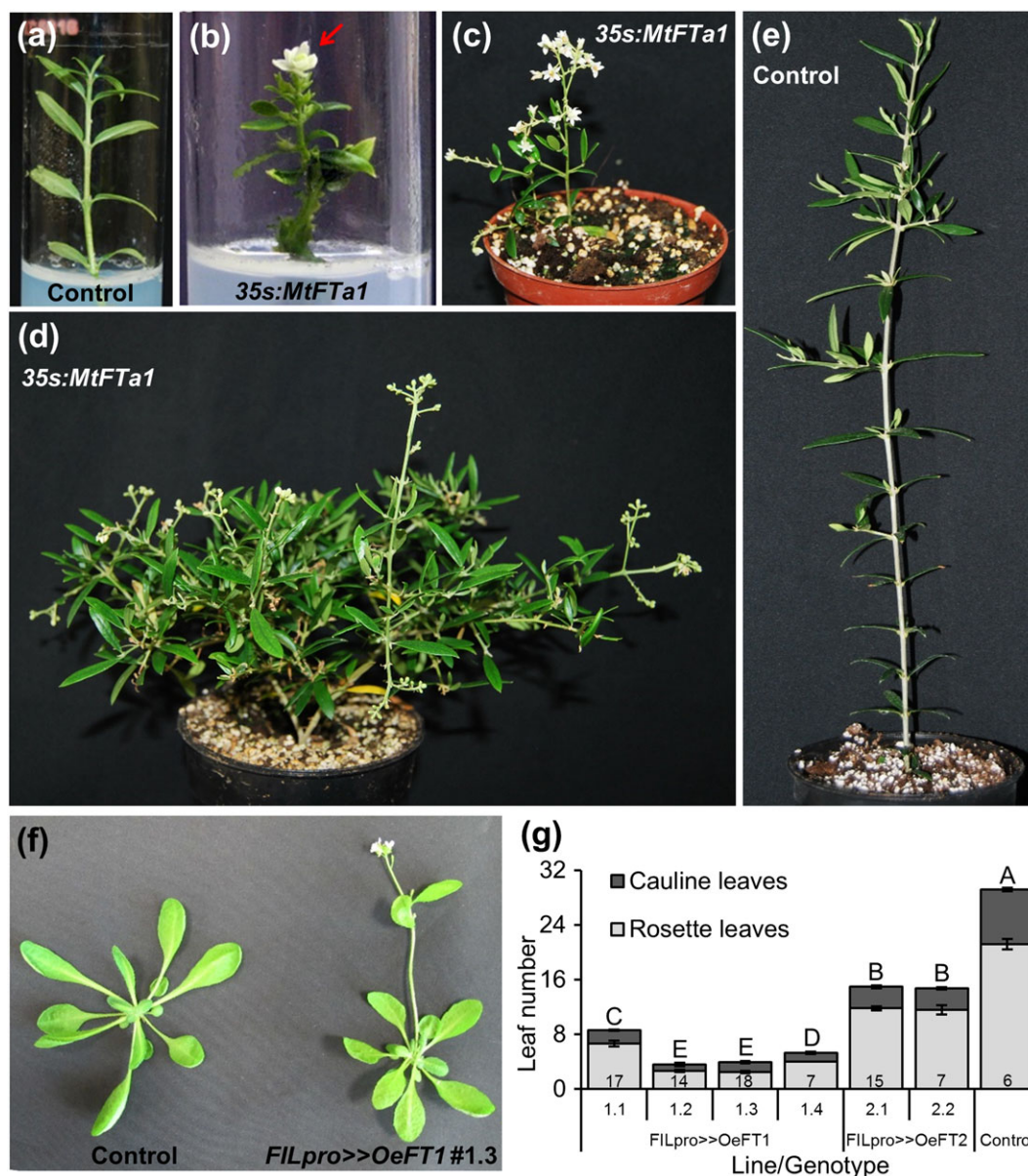


Figure 4. Early flowering of transgenic olive and *Arabidopsis* plants overexpressing FT-encoding genes. (a, b) Images of micro-propagated olive shoots from germinated embryos of control non-transgenic (a) and a 35s:MtFTa1-transgenic olive shoot (b, line FT5). Notice the terminal flower on the transgenic shoot (indicated by a red arrow), highlighting early loss of juvenility and the termination of the apical meristem with a single flower. (c, d) Flowering plants of transgenic line FT7 in the greenhouse 3 (c) and 9 (d) months after acclimatization to *ex vitro* conditions. (e) Control plant 9 months after acclimatization to *ex vitro* conditions. See Supporting Information Fig. S4 for additional images. (f) Images of 31-day-old *Arabidopsis* (Ler background) plants grown under short days, which delay flowering in *Arabidopsis*. The control *FILpro:LHG4* plant is still not flowering while the *FILpro>>OeFT1* plant has flowered after forming very few leaves. (g) Number of rosette and cauline leaves at flowering under short days of control *FILpro:LHG4* compared to *FILpro>>OeFT1* and *FILpro>>OeFT2* lines expressing olive FT-encoding genes. Numbers are mean values of independent biological repeats (plants) $\pm$ SE (bars). Number of repeats per line is presented in the column. Different letters represent significant differences between lines in total number of leaves according to Tukey–Kramer honestly significant difference (HSD) test on ranked data ($P \leq 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com]

the effect of cold temperature on olive flowering. In this round, we also followed the expression of the newly acquired molecular markers for flower induction – *OeFT1/2* expression in leaves. For controlled-environment experiments, we could not study trees that were of the same age as those studied in the field (10 to 14 years), owing to their size and immobility.

We thus studied 4- to 5-year-old ‘Barnea’ trees grown in 25 L pots (Materials and Methods section).

In an initial experiment, we used trees with a low fruit load to determine how shortening the period of exposure to cold winter temperatures would affect *OeFT1/2* expression and flowering. Control potted trees were exposed to a full natural

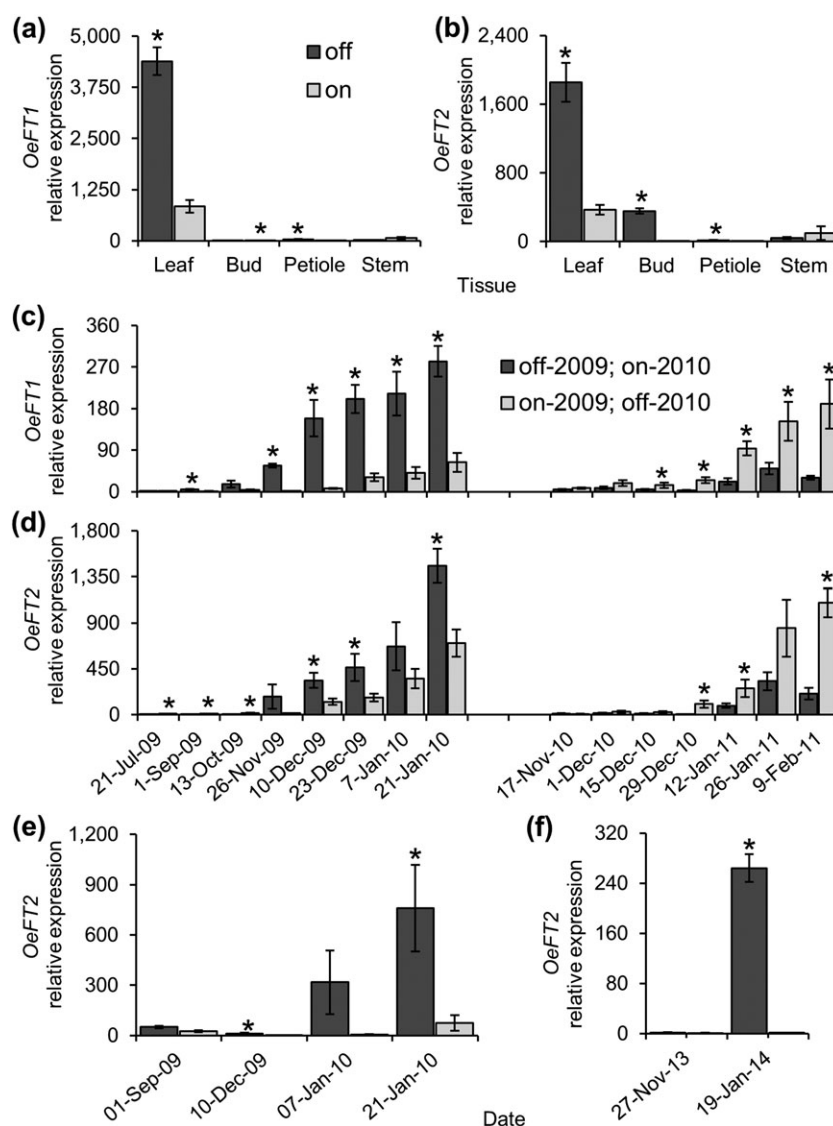


Figure 5. Relative expression of *OeFT1/2* in 'on' and 'off' trees. Samples were collected from 'on'-year or 'off'-year olive trees. (a, b) Relative expression of *OeFT1* (a) and *OeFT2* (b) on 19 Jan 2014 in leaf blade, leaf petiole, stem and lateral bud (see Supporting Information Fig. S2 for an image of the different tissues sampled). (c, d) Relative expression of *OeFT1* (c) and *OeFT2* (d) in leaves from trees during two seasons (2009–2011). Trees that were 'on' in the summer of 2009 were 'off' the following year. Trees that were 'off' in 2009 were 'on' in 2010. (e, f) Relative expression of *OeFT2* in stems with lateral buds during 2009–2010 (e) and isolated lateral buds in 2013–2014 (f). Relative expression was measured as described in Fig. 3. Numbers are mean values of three to five independent biological repeats (trees) $\pm$ SE (bars). Asterisks represent a significant difference between 'on' and 'off' trees in a specific tissue (a, b) or at a particular time point (c–f) according to Student's *t* test on ranked data ($P \leq 0.05$).

outdoor winter in a net house while we shortened the winter of treated trees by transferring them to a heated (minimum 15 °C) glasshouse (see Materials and Methods section for additional information) on either 15 Dec or 15 Jan.

In the net house, between 1 Nov and 15 Dec, trees were exposed for 237 h below 15 °C (see Materials and Methods section for additional information). Until 15 Jan and 1 Mar, hours accumulated below 15 °C reached 696 and 1340 h, respectively. In all trees, expression of both FT-encoding genes in leaves increased significantly from the end of October until 30 Dec (Fig. 7b,c). Expression of *OeFT1* was similar in all treatments in leaf samples taken from mid-January and the end of January (Fig. 7b). Expression of *OeFT2* in leaves was similar

in all treatments in samples taken in mid-January (Fig. 7c). However, by the end of January, the trees exposed to the shortest winter expressed lower levels of *OeFT2* compared to trees exposed to a full winter (Fig. 7c). Trees exposed to the shortest winter also had a significantly lower rate of flowering (11%) compared to trees moved to the warmed glasshouse in mid-January (29%) or kept all winter in the net house (43%; Fig. 7a). It might be that the extra 459 h below 15 °C between 15 Dec and 15 Jan is important to maintaining high levels of *OeFT2* in the leaves, and this in turn influences the degree of flower induction. On the other hand, proper flower induction in olive may require a period of at least 75 d on which temperatures reach levels below 15 °C.

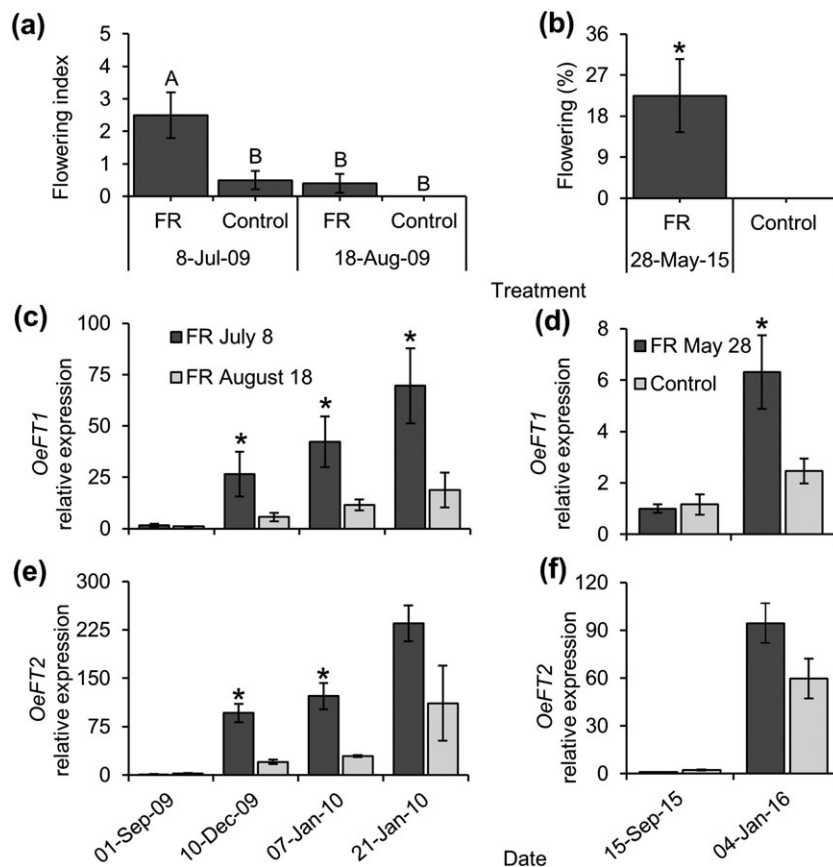


Figure 6. Flowering and relative expression of *OeFT1/2* in leaves from tree limbs after early fruit removal. All fruitlets were removed (FR) from one of the limbs of 'on' trees, leaving similar limbs with heavy fruit load until harvest. Fruitlet removal was performed on 8 Jul or 18 Aug 2009 (a, c, e) and on 28 May 2015 (b, d, f). (a) Flowering index was estimated in the following season (2010) in the treated (FR) and control limbs, as described in Fig. 1. (b) Percent of lateral buds forming inflorescences in the spring in pre-selected branches ($n = 7-19$); buds on the first three nodes of recent seasonal vegetative growth were scored (see red square frame in Fig. 1). (c–f) Relative expression of *OeFT1* (c, d) and *OeFT2* (e, f) in leaves, measured as described in Fig. 3. Numbers are mean values of three to five independent biological repeats (tree limbs) $\pm$ SE (bars). (a) Different letters represent significant differences according to Tukey–Kramer honestly significant difference (HSD) test ($P \leq 0.05$). (b–f) Asterisks represent a significant difference between treatments at the same time point according to Student's *t* test ($P \leq 0.05$). (b, c, e) The statistical test was performed on ranked data.

Cold winter temperatures are required for increased expression of FT-encoding genes and subsequent flowering

We then explored the outcome of replacing natural winter conditions with different controlled-temperature regimes. The first set of experiments was begun on 25 Nov 2014 with 'off' trees in pots. Control trees were subjected to natural winter conditions in a net house. Treated trees were moved to a glasshouse with controlled-environment conditions (phytotron, day/night of 9/15 h, respectively, Materials and Methods section). Trees were exposed for 89 d (until 22 Feb 2015) to one of two specific temperature regimes: 16/10 °C day/night or 28/22 °C day/night. As expected, control 'off' trees exposed to natural winter conditions produced many inflorescences the following spring (51% of lateral meristems; Fig. 8a). A significantly higher (86%) rate of lateral meristems flowered in trees exposed to the 16/10 °C day/night regime. In contrast, the 'off' trees transferred to the 28/22 °C day/night regime did not produce any inflorescences the following spring (Fig. 8a). The inflorescences in the trees exposed to the 16/10 °C day/night regime already

appeared (visible to the naked eye) towards the end of January, ~60 d from the beginning of the cold treatment, while still under the cold-temperature regime. Inflorescences on control trees appeared 1–2 months later. This would imply that a cold-temperature regime shorter than 89 d is sufficient for this degree of flowering. The complete lack of flowering under the 28/22 °C day/night regime clearly demonstrates the need for cold temperatures during winter to achieve flowering in olives.

We studied the expression of *OeFT1* in leaves and *OeFT2* in both leaves and lateral buds, 42 d (6 Jan) and 72 d (5 Feb) from the beginning of the treatments. Expression of these genes increased significantly from the beginning of the experiment in trees exposed to natural winter temperatures or to the 16/10 °C day/night regime (Fig. 8b–d). Expression of both genes was significantly lower in trees exposed to the 28/22 °C day/night regime. Expression of *OeFT2* in leaves at the beginning of February best mirrored the flowering response: highest in trees exposed to the 16/10 °C day/night regime, slightly but significantly lower in trees exposed to natural winter conditions and basal in trees exposed to the 28/22 °C day/night regime

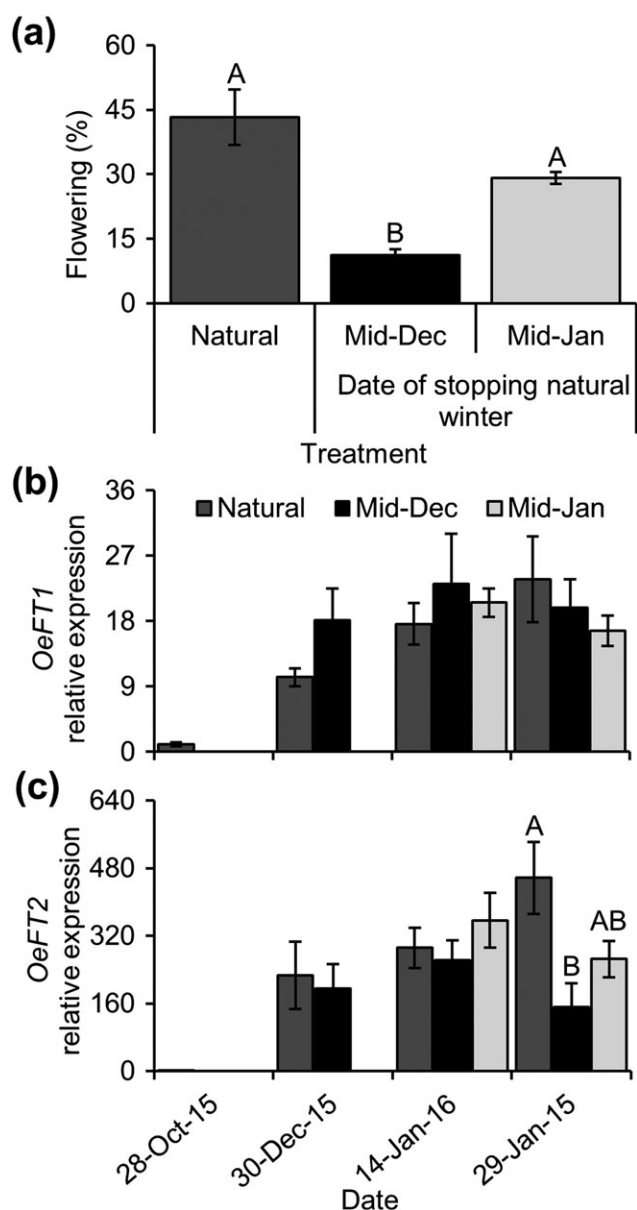


Figure 7. Flowering and relative expression of *OeFT1/2* in trees exposed to a shortened winter season. Trees in pots were kept in a net house with natural outdoor winter conditions. On 15 Dec (designated Mid-Dec) or 15 Jan (Mid-Jan), treated trees were transferred to a heated glasshouse (minimum 15 °C) and kept there until 15 Mar (see Materials and Methods section for additional information). Control trees were kept under natural conditions throughout the experiment. (a) Percent flowering the following spring (2016) on pre-selected branches ($n = 8$), measured as described in Fig. 6. (b, c) Relative expression of *OeFT1* (b) and *OeFT2* (c) in leaves. On 28 Oct, all trees were under natural conditions. On 30 Dec, we measured trees in natural conditions and trees that were moved to the greenhouse on 15 Dec. On 14 Jan, we measured trees in natural conditions, trees that were moved to the greenhouse on Dec 15 and trees that will be moved to the greenhouse on 15 Jan. Relative expression was measured as described in Fig. 3. Numbers are mean values of four independent biological repeats (trees) $\pm$ SE (bars). Different letters represent significant differences between treatments at the same time point according to Tukey–Kramer honestly significant difference (HSD) test ($P \leq 0.05$).

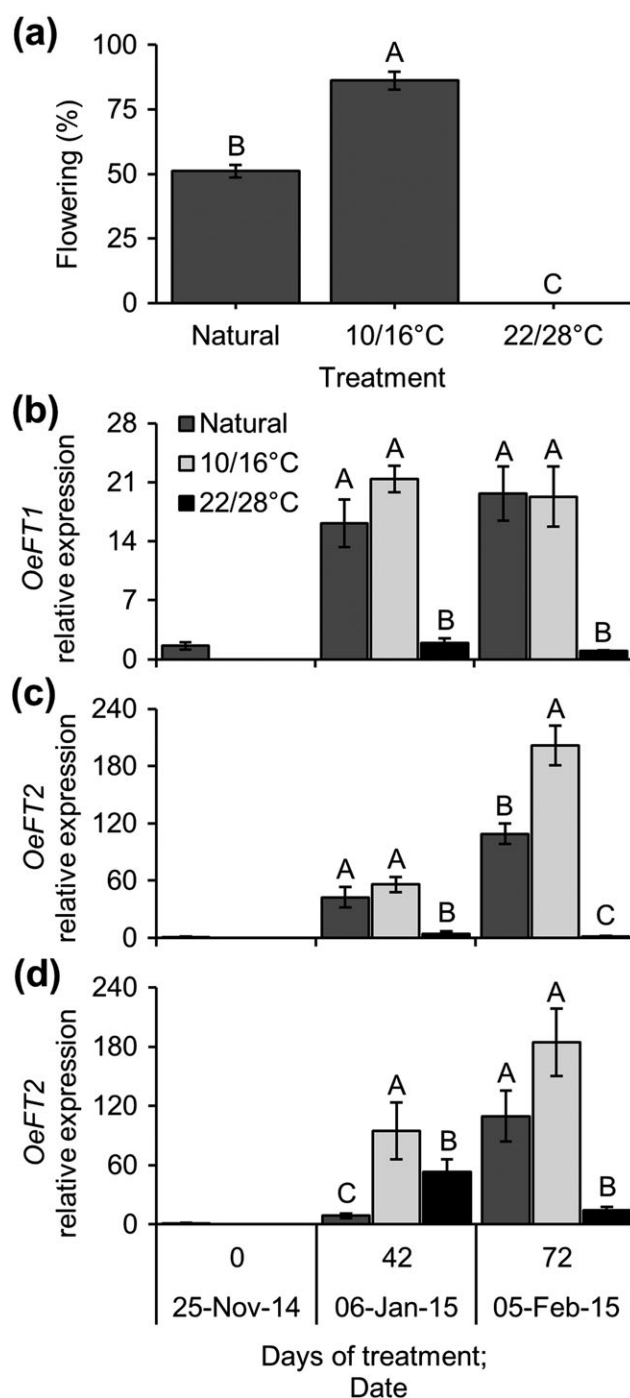


Figure 8. Flowering and relative expression of *OeFT1/2* in trees exposed to different temperatures during the winter. Trees in pots were kept in a 'phytotron' from 25 Nov 2014 to 22 Feb 2015 (89 d), under a temperature regime of 10/16 °C or 22/28 °C (night/day). Additional similar trees were kept under natural conditions. (a) Percent flowering the following spring (2015) on pre-selected branches ($n = 10$), measured as described in Fig. 6. (b–d) Relative expression of *OeFT1* (b) and *OeFT2* (c, d) in leaves (b, c) or stems containing lateral buds (d). Relative expression was measured as described in Fig. 3. Numbers are mean values of four independent biological repeats (trees) $\pm$ SE (bars). Different letters represent significant differences between treatments at the same time point according to Tukey–Kramer honestly significant difference (HSD) test on ranked data ($P \leq 0.05$).

(Fig. 8c). These results show that without cold temperatures, there is no increase in *OeFTI/2* levels during winter and no flowering. This suggests that *OeFTI/2* transcript accumulation in leaves during the winter is required for floral transition and without such accumulation, the lateral meristems remain vegetative.

Out-of-season cold temperatures increase the expression of FT-encoding genes but degree of flowering depends on additional factors

Exposing olive trees to an artificial chilling period causes out-of-season flowering (Hartmann and Whisler 1975). We wanted to repeat these experiments to determine whether a cold-dependent increase in *OeFTI/2* expression is limited to a certain time of year, or perhaps dependent on the developmental age of the leaf exposed to the cold. To test this, potted 'off' trees were transferred to the 16/10 °C day/night regime starting on 22 Jun or 1 Sep.

The control trees exposed to outside conditions had, as expected, baseline levels of *OeFTI/2* in leaves on both 18 Aug and 28 Oct. In contrast, trees exposed to the 16/10 °C day/night regime for 57 d expressed high levels of *OeFTI/2* in leaves on those dates (Fig. 9b,c). *OeFTI/2* levels were similar to those detected for trees after exposure to a natural winter (on 15 Jan). Thus, cold temperatures caused an increase in *OeFTI/2* expression in leaves independent of leaf developmental age or time of year.

The treated trees were exposed to the 16/10 °C day/night regime for 70 d, and the rate of flowering in lateral buds was measured a month later. As expected, control trees kept outside produced no new inflorescences during the fall or winter and flowered normally the following spring. On the other hand, trees exposed to the 16/10 °C day/night regime starting 22 Jun had 13% lateral buds with visible inflorescences by mid-September (Fig. 9a). Trees exposed to the 16/10 °C day/night regime starting 1 Sep 2015 reached 55% lateral buds with visible inflorescences by the beginning of December (Fig. 9a). These inflorescences were slower to emerge than those from the summer treatment, probably because of the lower temperatures in autumn.

This experiment clearly showed that out-of-season cold temperatures can cause flowering in olive. Still, the degree of flowering was significantly weaker after the June treatment. This weaker flowering response did not correlate with leaf *OeFTI/2* levels 57 d from the beginning of the treatment, which were similar for the June and September exposures (Fig. 9b,c). We repeated the June cold treatment on other trees the following year, and this again induced a relatively low degree (~12%) of out-of-season flowering (Supporting Information Fig. S8a). *OeFTI/2* levels in leaves of treated trees were high, as in the previous year, and similar to trees treated in November (Supporting Information Fig. S8b,c). While high *OeFTI/2* levels in leaves seemed to lead to flower induction in nearby lateral meristems, this latest finding suggests that additional factors modulate the flowering response.

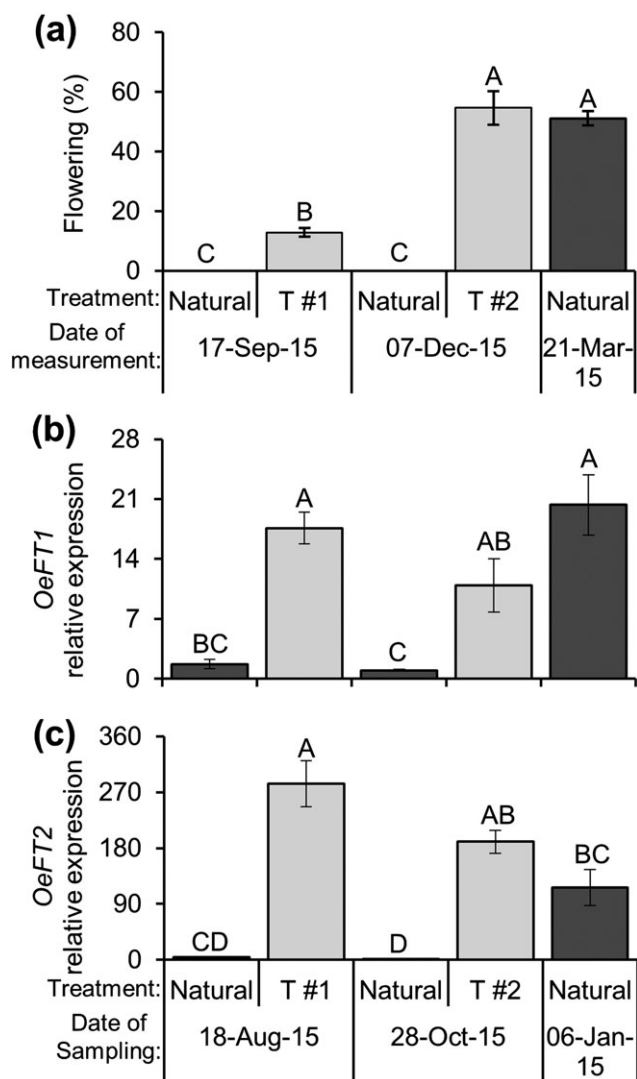


Figure 9. Effect of exposure to cold temperatures before winter time. Potted trees were transferred to a controlled environment of 16/10 °C (day/night) for 70 d on 22 Jun 2015 (designated T #1) and 1 Sep 2015 (T #2). Control potted trees were kept outdoors (natural). (a) Percent flowering following the treatment, or control, on pre-selected branches ($n = 10\text{--}16$), measured as described in Fig. 6. Percent flowering was measured at three different dates, as designated. (b, c) Samples for gene expression analysis were taken 57 d from the start of the treatment period and compared to trees kept under natural outdoor conditions. Relative expression of *OeFTI* (b) and *OeFTI2* (c) in leaves measured as described in Fig. 3. Numbers are mean values of four independent biological repeats (trees) $\pm$ SE (bars). Different letters indicate a significant difference according to Tukey–Kramer honestly significant difference (HSD) test on ranked data ($P \leq 0.05$).

An increase in the expression of a TFL1-encoding gene in response to previous fruit load

Recent findings in apple suggest that TFL1-encoding genes may mediate the flowering response to season and fruit load (Haberman et al. 2016). The olive genome (Cruz et al. 2016) contains three genes encoding proteins similar to *Arabidopsis* and apple TFL1 (Supporting Information Data S1). We were

only able to detect the expression of one of the genes, *OeTFL1-1*, in lateral buds and not in leaves. Levels of *OeTFL1-1* in stems with lateral buds were similar in trees subjected to artificial winter in June compared to November (Fig. 10a). Thus, the pattern of *OeTFL1-1* expression cannot explain the reduced flowering in June versus November cold treatments. Interestingly, results from two seasons showed that *OeTFL1-1* transcripts accumulate to significantly higher levels in mid-January in lateral buds of 'on' trees compared to 'off' trees (Fig. 10b,c). Thus, it is possible that *OeTFL1-1* transcript accumulation in olive lateral buds in response to 'biochemical memory' of fruit load modulates the meristem's response to cold-induced *OeFTI2* accumulation.

DISCUSSION

We did not find evidence supporting the hypothesis (Rallo and Martin 1991) that formation of an olive inflorescence occurs naturally before the winter. Under local conditions (northern hemisphere, Israel), 'Barnea' olive inflorescences form in lateral meristems in late January–February. We base this conclusion on our SEM analysis of lateral meristems as well as on the expression of a putative inflorescence identity gene (*OeAPI-1*) and organ identity gene (*OeAG-1*). Supporting this, sections of lateral buds from 'Leccino' and 'Puntino' grown in Pisa, Italy, showed no inflorescence formation until March (Andreini *et al.* 2008), and 'Manzanillo' in California showed no inflorescence formation until April (Badr *et al.* 1970).

In our findings, as well as those of Hartmann and Whisler (1975), it is only when potted trees were subjected to artificial cold treatments in early summer (June) or fall (September) that inflorescence formation could occur before the winter season. These initial findings suggested that cold temperatures are likely required for floral induction in olives. We then showed that ectopic expression of an FT-encoding gene causes early flowering (loss of juvenility) in transgenic olives, suggesting that, similar to other species, FT regulates flowering in olive. We identified two FT-encoding genes in the olive genome and provided evidence of a dramatic increase in both genes' expression during the winter or by artificial cold-temperature treatments in other seasons. We showed that these genes encode functional FT proteins capable of causing early flowering in *Arabidopsis*, under the regulation of a leaf-specific promoter. Thus, transcript accumulation in olive leaves (*OeFTI2*) and lateral meristems (*OeFT2*) towards the end of winter is likely a major event in olive flower induction. Together, these findings clearly suggest that in 'Barnea' under local conditions, flower induction occurs during the winter because of cold-temperature induction of FT-encoding genes, and inflorescence formation/initiation occurs in lateral buds at the end of winter.

While we cannot rule out the option that in other cultivars grown in other Mediterranean climates, flower induction occurs in midsummer (Navarro *et al.* 1990; Pinney and Polito 1990), this appears highly unlikely because, as far as we know, all olive cultivars require some degree of cold temperature to flower.

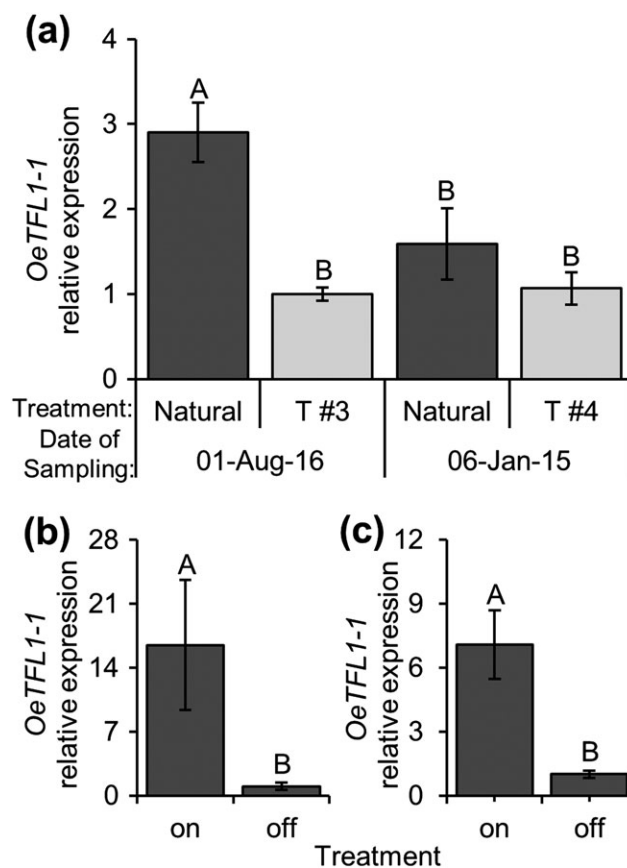


Figure 10. Relative expression of *OeTFL1-1* prior to flower initiation. (a) Potted trees were transferred to a controlled environment of 10/16 °C (night/day) for 70 or 89 d on 22 Jun 2016 (designated: T #3) or 25 Nov 2014 (T #4), respectively. Control potted trees were kept outdoors (natural). Samples for gene expression were taken 40–42 d from the start of each treatment and compared to outside trees on the same day. (b, c) Samples were collected from 'on' and 'off' trees on 26 Jan 2011 (b) and 19 Jan 2014 (c). Relative expression of *OeTFL1-1* in stems containing lateral buds (a) or lateral buds (b, c) measured using a TaqMan probe (a, b) or Syber Green (c). Numbers are mean values of four (a, b) or five (c) independent biological repeats (trees) ± SE (bars). Different letters indicate a significant difference according to Tukey–Kramer honestly significant difference (HSD) test (a; $P \leq 0.05$) or Student's *t* test on ranked data (b, c; $P \leq 0.05$).

Cold temperatures are required for dormancy release in deciduous trees, and in some examples, such as apples, inflorescences are formed before winter, in summer (Haberman *et al.* 2016). Such studies encouraged scientists to propose that a similar process occurs in olives (Rallo and Martin 1991; Fabbri and Bennelli 2000), although no clear sign of inflorescence differentiation was shown to occur before the end of winter. Our results suggest that cold winter temperatures cause an increase in FT expression, and we provide supporting evidence suggesting this is linked to flower induction. Still, cold temperatures might also contribute to release of lateral bud dormancy in olive, and one or both of the FT-encoding genes might be mediating this process as well. There is some evidence that FT-encoding genes have a role in the control of bud dormancy in other perennial species (Hsu *et al.* 2011; Freiman *et al.* 2015). Our finding that the transgenic olives overexpressing MtFTa1 lost apical

dominance causing growth of many lateral buds might support such a theory.

Interestingly, while the *OeFT1/2* levels reached after artificial cold-temperature regime seemed to be similar in different months, the degree of flowering was quite different. Perhaps seasonal internal factors inhibit FT function. *OeTFL1-1* did not seem to be a good candidate, as its expression was similar in trees exposed to cold temperatures in different months. Examples in *Arabidopsis* of additional repressors of FT function are the ectopically expressed FWA protein (Soppe *et al.* 1999; Ikeda *et al.* 2007), brother of FT and TFL1 (BFT) protein that accumulates under high salinity (Ryu *et al.* 2014) and branched 1 in axillary buds (Niwa *et al.* 2013). The response of lateral buds to high levels of FT might also be influenced by events within the shoot, such as changes in the dominance/activity of the apical meristem. In summer, movement of sugars and other macromolecules produced in the leaves may be directed inclusively to the actively growing apical bud rather than the quiescent lateral buds.

We show here that olive juvenility is dramatically shortened in transgenic plants in which an FT-encoding gene is overexpressed. These transgenic plants also flower out of season, as they are no longer dependent on cold-temperature induction. Still, flowering seems to be more pronounced in the winter.

Ectopic expression of FT can cause a shortened juvenile phase as well as out-of-season flowering in several transgenic perennials (Endo *et al.* 2005; Trankner *et al.* 2010). Overexpression of other floral integrators such as LEAFY and APETALA1 could also shorten juvenility (Pena *et al.* 2001), perhaps with less consistent efficiency in different species (Rottmann *et al.* 2000; Strauss *et al.* 2004; Hanke *et al.* 2007). In any given summer, a typical commercial olive orchard contains trees of the same cultivar, a majority/minority with HFL, while the rest carry very few fruits. In rare cases, one or a few of the branches have different fruiting behaviour from the rest of the tree. The olive is relatively sectorial, meaning that branches may show some degree of autonomous behaviour in flowering (Sprugel *et al.* 1991; Lavee 2007). Here we studied two branches from the same tree, one carrying an HFL and the other with no fruit (all fruitlets removed in late May). After the winter, the new lateral meristems on the branch treated with early fruitlet removal formed inflorescences, while no inflorescences formed on the control branch that carried fruit until harvest. As already noted, this is partially due to a severe reduction in growth, leading to much fewer new lateral buds formed on the branch. There is a clear trade-off between fruit and vegetative growth in olive (Bustan *et al.* 2016). Here we provide evidence that the few lateral meristems formed during an 'on' year do not produce inflorescences. This was also the case in all other trees from the different experiments carrying an HFL.

Evidence for an important flowering-related event in the summer, which sparked the summer induction theory, is as solid now as it was in the past: up to a certain time, the inhibitory effect of fruit load on flowering can be reversed by fruit removal. Without molecular markers such as FT expression, it was difficult to set a timeline for olive flower induction in the

1990s, and this summer event was referred to as flower induction (Fabbri and Bennelli 2000). Here, studying FT expression, we observed that fruit-dependent changes still occur when fruitlets are removed as early as August. Later fruit removal no longer restores FT expression or the next year's flowering. Yet, the number of nodes formed by the shoot was not significantly different between the two fruit removal treatments (Supporting Information Fig. S7). We refer to events in summer as establishing a 'biochemical memory' of fruit load. Once established, it no longer requires the fruits' presence to affect flower induction in the winter. This 'biochemical memory' causes a significant reduction in winter *OeFT1/2* accumulation in leaves, and no expression of *OeFT2* and higher levels of *OeTFL1-1* in lateral buds. These changes in gene expression occur two or more months after fruit harvest. Thus, unlike that in apples (Haberman *et al.* 2016), citrus (Shalom *et al.* 2012) and avocado (Ziv *et al.* 2014), flower induction in olives occurs at a time when, normally, no fruit are present on the tree. The nature of this 'biochemical memory' is unknown to us, yet its outcome may be through *OeFT* and *OeTFL1-1* gene expression.

The source of the signal that forms the 'biochemical memory' might be the seed (Stutte and Martin 1986). It might include histone modifications, as shown to be crucial in preserving the 'memory' of vernalization in *Arabidopsis* (Berry and Dean 2015; Lee *et al.* 2015). The range of inductive cold temperatures in olive is different (higher temperatures) from those that induce a vernalization response in *Arabidopsis* or *Arabis alpina* (Bergonzi *et al.* 2013).

Hormones such as gibberellin may be involved, as was recently suggested in apple (Haberman *et al.* 2016) and in many other species (Wilkie *et al.* 2008). There appears to be an increase in gibberellic acid (GA) activity in olive lateral buds during winter (Badr *et al.* 1970). Findings in 'Memecik' olive suggested that GA₃ levels are higher in the lateral buds of trees with HFL (compared to trees with no fruit) in both July and November. On the other hand, levels of GA₄ were lower in these buds than in those taken from trees with no fruit (Ulger *et al.* 2004). In November, levels in leaves of certain microRNAs are affected by HFL (Yanik *et al.* 2013).

In citrus (Muñoz-Fambuena *et al.* 2011), mango (Nakagawa *et al.* 2012) and avocado (Ziv *et al.* 2014), the level of FT expression during the winter is also increased in trees with no fruit but, unlike olive, is minimal to non-existent in trees with HFL. A similar qualitative difference in expression was found here in olive when measuring *OeFT2* in lateral buds. *OeFT2* was only expressed in lateral buds of trees with no 'biochemical memory' of fruit load. There is a possibility that leaf-derived *OeFT1/2* does not reach lateral buds; thus, only *OeFT2* accumulation in these buds triggers flowering.

Expression of *OeFT2* was much higher in leaves. The transcript found in buds was likely formed in the bud because intermediate tissues (petiole and stem) displayed little or no *OeFT2* expression, and the FIL promoter used in our transgenic *Arabidopsis* studies is not expressed in the meristem (Lifschitz *et al.* 2006). *Arabidopsis* plants containing the *OeFT1* construct flowered much earlier than those with the *OeFT2* construct. This did not seem to be due to higher levels of transcript for *OeFT1*. It could be that *OeFT2* movement in the phloem is less

efficient than that of OeFT1; this would explain why it causes less early flowering in *Arabidopsis* when expressed under the FIL promoter. A difference in movement efficiency has been shown between FT and TSF proteins (Jin *et al.* 2015). If indeed OeFT2 movement is less efficient, this might explain why it is expressed directly in olive lateral buds. Studying the effect of OeFT1/2 ectopic expression in transgenic olives using different promoters would help clarify these points.

The requirements for sufficient cold temperatures (period length or temperature) for olive flowering seem to vary among olive genotypes (Hartmann and Porlingis 1957; Hartmann and Whisler 1975; Aybar *et al.* 2015). Cultivars originating from colder regions in Europe barely flower under warmer winters (Aybar *et al.* 2015). Because winters are progressively becoming warmer (IPCC 2014), the future of olive culture might largely depend on identifying genotypes that flower with less cold requirements.

ACKNOWLEDGEMENTS

We dedicate this work to the late Prof. Shimon Lavee who introduced us all (and many others worldwide) to olive biology. Shimon helped design the experiments and interpret the results. He will be greatly missed. We thank the anonymous reviewers for their helpful comments. We thank Ravit Eshed and Eviatar Steiner for their excellent advice on strategies for construct preparation for plant transformation, Benjamin Avidan and Oren Osterseizer-Biran for their support at the beginning of this project and the team of 'Gadash Tsabar Kama' for providing access to trees in their commercial orchard. The research is funded by the Israeli Science Foundation – the Charles H. Revson Foundation (grant no. 1464/07) to A.S., the Chief Scientist Ministry of Agriculture and Rural Development, Israel (grants no. 837-0132-11 and no. 12-02-0002) to A.S. and by grant P11-AGR 7992, Junta de Andalucía, Spain to F. P.-A.

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Received 1 November 2016; received in revised form 3 January 2017; accepted for publication 4 January 2017

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 Pictures of trees in the experiments. (a–c) 'Barnea' olive trees in 25 L pots on 29 Mar 2015. (a) A tree that was kept from 25 Nov 2014 to 22 Feb 2015 (89 d) under a temperature regime of 22/28 °C (night/day). It is impossible to find inflorescences on the tree. (b) A tree similar to (a) that was exposed to a temperature regime of 10/16 °C. Numerous inflorescences can be seen on the tree. (c) Comparison of both trees. (d) 'Barnea' olive tree in the orchard where experiments were conducted. Based on previous studies (Lavee and Wodner 2004), a 9-year-old 'Barnea' 'on' tree grown at a density of 300 trees per hectare produces ~70 kg fruit, each fruit weighing on average 1.80 g. Thus, such a tree carries ~39 000 fruit. One-year-old 'Barnea' shoots from 'on' trees with 35–95% lateral buds forming inflorescences produce 5.8–7.1 fruit per shoot (Lavee *et al.* 1999). This means that such a tree, during the previous 'off' year, likely produced 5000–7000 new growth shoots. We measured the average number of inflorescences per new growth shoot in the orchard and it was ~20 inflorescences per

shoot. Thus, we predict an 'on'-year tree forms ~100 000–140 000 inflorescences. A 'Barnea' 'off' tree grown in the same density could produce ~10 kg fruit, each fruit weighing on average 4.00 g (Lavee and Wodner 2004). Thus, such a tree carries ~2500 fruit. Fruit set is higher in 'off' trees (Suarez *et al.* 1984), so such a tree likely forms less than 6000 inflorescences.

Figure S2 Tissues in the experiments. (a, b) Pictures of olive shoots. The red arrow marks the postulated first node of the new vegetative growth in spring. (b) The shoot segment sampled and analysed for gene expression, SEM imaging and flowering is cut from the rest of the shoot. (c) The leaves are cut at the petiole and separated from the stem, leaving a stem carrying lateral buds. (d) The buds and petioles are separated from the stem, leaving separate buds, petioles and stem. (e) An enlarged image of separated buds.

Figure S3 Expression levels of the *MtFta1* gene in olive embryogenic lines. The expression levels of *MtFta1* in different transgenic olive embryogenic lines were measured by quantitative real-time RT-PCR. As expected, no expression was detected in non-transgenic control callus (not shown). Relative expression values were normalized to the line with the lowest expression (FT12). Transgenic lines FT7 and FT15 expressed highest *MtFta1* mRNA levels. Both lines, together with line FT5, flowered under *in vitro* culture. Black arrows indicate embryogenic lines that gave rise to flowering plants under *in vitro* culture. The time of transition to flowering differed among plantlets obtained from the same paromomycin-resistant mass (independent transgenic line). All germinated plants from line FT15 flowered during the *in vitro* culture, while only 44 and 40% of plants from lines FT7 and FT5 flowered *in vitro*, respectively.

Figure S4 Early flowering in transgenic olive plants overexpressing the *MtFta1* gene. (a, b) Same images as those shown in Fig. 4a,b. (a) Control non-transgenic *in vitro* grown shoot. (b) Solitary flower on *in vitro* grown shoot from transgenic line FT5, 15 d after completion of the somatic embryo germination phase. (c) Grouped flowers on *in vitro* grown shoot from the FT7 line. Commonly, lateral shoots ended up producing solitary or clustered flowers, inducing the development of secondary axillary shoots. (d) *In vitro* flowering shoot showing reduced growth and necrosis following germination. (e) Growth habit during *in vitro* culture of FT7 shoots, showing development of lateral shoots and apical growth cessation (white arrows indicate floral buds or developed flowers) and (f) control shoots showing apical dominance. (g, h) Flowering plant from transgenic line FT7 3 months (g) and 4 months (h) after acclimatization, and (i) control non-transgenic plant after acclimatization. Scale bars correspond to 1 cm in (a–f) and 2 cm in (g–i).

Figure S5 Molecular phylogenetic analysis of FT/TFL1 encoding proteins. The tree was constructed with the predicted protein sequences of olive: OeFT1 (OE5A107414T1), OeFT2 (OE5A103537T1), OeTFL1-1 (OE5A037908T1), OeTFL1-2 (OE5A094303T1), OeTFL1-3 (OE5A077048T1); *Arabidopsis*: AtFT (AT1G65480.1), AtTSF (AT4G20370.1), AtTFL1 (AT5G03840.1); and *Medicago*: MtFta1 (HQ721813.1). The evolutionary history was inferred by using the maximum likelihood method based on the JTT matrix-based model (Jones *et al.* 1992). The tree with the highest log-likelihood (−1726.1325) is shown. Initial tree for the heuristic search was obtained automatically by applying neighbour-join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model and then selecting the topology with

superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved nine amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 170 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.* 2016).

Figure S6 Relative expression of *OeFT1/2* in transgenic *Arabidopsis*. Wild-type (WT) plants (Fil;LhG4) were transformed with a construct containing *OeFT1* or *OeFT2*. (a) PCR with primers for the transgenic insert, on DNA from the T1 *Arabidopsis* transgenic lines, loaded on a 1% agarose gel. The presence of the insert can be seen in the transgenic lines' DNA and is absent from the WT control (Fil;LhG4). (b) Relative expression of *OeFT1/2* in the above-ground part of 15-day-old T3 *Arabidopsis* seedlings, descendants of specific T2-transformed plants that constitutively expressed *OeFT1* or *OeFT2*. Relative expression was measured as described in Fig. 3, with primers that amplify both inserts, *OeFT1* and *OeFT2*. Numbers are mean values of four independent biological repeats (each repeat is 10 seedlings collected and combined for a single sample) $\pm$ SE (bars). Different letters represent significant differences according to Tukey–Kramer HSD test ($P \leq 0.05$).

Figure S7 Vegetative growth of shoots from tree limbs after early fruit removal. All fruitlets were removed (FR) from one of the limbs of five 'on' trees, leaving similar limbs with heavy fruit load until harvest. Fruitlet removal was performed on 8 Jul or 18 Aug 2009. Shoot node number (a, c) and length (cm; b, d) were measured in pre-selected shoots ($n = 3$) of FR limbs, as well as five 'on' and 'off' trees. Measurement was from the postulated first node of the current year's vegetative growth (from last spring) until the last node separated from the apical bud by a clear internode (at least 1 mm in length; a, c) or the tip of the shoot (b, d). (a, b) Last measurement on 7 Jan 2010. (c, d) Two-week interval measurements from 8 Jun 2009 to 7 Jan 2010. Red arrows in (c, d) indicate the date of the fruitlet removal. Final length was significantly longer, and final node number was significantly higher in shoots from 'off'

trees compared to shoots of 'on' trees. The earlier 8 Jul fruit removal significantly increased shoot node number compared to 'on' tree shoots. Numbers are mean values of five independent biological repeats (tree limbs) $\pm$ SE (bars). (a, c) Different letters represent significant differences according to Tukey–Kramer HSD test on ranked data ($P \leq 0.05$).

Figure S8 Exposure to cold temperatures before winter time in an additional experiment. Potted trees were transferred to a controlled environment of 16/10 °C (day/night) for 70 d on 22 Jun 2016 (designated T #3). Additional similar potted trees were kept under natural conditions as a control (natural). Gene expression was compared to potted trees exposed to a similar treatment in 25 Nov 2014 (T #4), for which results were already presented in Fig. 6. (a) Percent of flowering following the treatment, or control in pre-selected branches ($n = 10$ –12), measured as described in Fig. 6. As expected, control (natural) trees grown outdoors (dark grey bar) flowered after winter. (b, c) Samples for gene expression were taken 40–42 d from the start of the 16/10 °C treatment period and compared to trees kept under natural conditions at similar dates. Relative expression of *OeFT1* (b) and *OeFT2* (c) in leaves, measured as described in Fig. 3. Numbers are mean values of 4 independent biological repeats (trees) $\pm$ SE (bars). Different letters indicate a significant difference according to Tukey–Kramer HSD test ($P \leq 0.05$).

Figure S9 Validation of *OeACT7-1* as a reference gene for qPCR. Relative read number (coverage) of *OeACT7-1* (OE5A117728T2) compared to that of the β -actin gene (OE5A087678T4), which was tested and validated as an appropriate reference gene in olive (Dündar *et al.*). RNA-Seq data of 10 samples, obtained from the olive genome data (Cruz *et al.*), were compared. Read number was acquired at the coordinate Oe5_s00163:375915 for *OeACT7-1* and Oe5_s02010:364726 for the β -actin gene. This coordinate constitutes a conserved methionine at position 285 in the predicted protein. The change in relative expression of both genes between different tissues and treatments seems similar.