Bud sprouting and floral induction and expression of *FT* in loquat [*Eriobotrya japonica* (Thunb.) Lindl.]

Carmina Reig[1], Francisco Gil-Muñoz[1], Francisco Vera-Sirera[2], Ana García-Lorca[1], Amparo Martínez-Fuentes[1], Carlos Mesejo[1], Miguel A. Pérez-Amador[2] and Manuel Agustí[1]*

[1]Instituto Agroforestal Mediterráneo, Universitat Politècnica de València, Valencia, Spain.
[2]Instituto de Biología Molecular y Celular de Plantas, CSIC-UPV, Valencia, Spain

***Corresponding author**:

Manuel Agustí
magusti@prv.upv.es
Phone: +34 963879330
Fax: +34 963877339

Abstract

***Main conclusion EjFT1* and *EjFT2* genes were isolated and sequenced from leaves of loquat. EjFT1 is related to bud sprouting and leaf development, *EjFT2* is involved in floral bud induction.**

Loquat [*Eriobotrya japonica* (Thunb.) Lindl.] is an evergreen species belonging to the family Rosaceae, such as apple and pear, whose reproductive development, in contrast with these species, is a continuous process that is not interrupted by winter dormancy. Thus, the study of the mechanism of flowering in loquat has the potential to uncover the environmental and genetic networks that trigger flowering more accurately, and, hence, contributing for a better understanding of the Rosaceae floral process. As a first step toward understanding the molecular mechanisms controlling flowering, extensive defoliation and defruiting assays, together with molecular studies of the key *FLOWERING LOCUS T* (*FT)* gene, were carried out. *FT* exhibited two peaks of expression in leaves, the first one in early to mid-May, the second one in mid-June. Two *FT* genes, *EjFT1* and *EjFT2*, were isolated and sequenced and studied their expression. Expression of *EjFT1* and *EjFT2* peaks in mid-May, at bud sprouting. *EjFT2* expression peaks again in mid-June, coinciding with the floral bud inductive period. Thus, when all leaves of the tree were continuously removed from early- to late-May vegetative apex differentiated into panicle, but when defoliation was performed from early- to late-june apex did not differentiate. On the other hand, fruit removal advanced *EjFT1* expression in old leaves and the sooner the fruit detached, the sooner the bud sprouted. Accordingly, results strongly suggest that *EjFT1* might be related to bud sprouting and leaf development, while *EjFT2* might be involved in floral bud induction. An integrative model for *FT* functions in loquat is discussed.

Key words Rosaceae, *EjFT1*, *EjFT2*, , flowering, fruit trees, perennials.

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Introduction

The major fruit tree species from a commercial point of view belong to the family Rosaceae, subfamily Amygdaloideae, that include, among others, apple, pear, quince, or Japanese pear. According to FAO, the world produced 110x10^6 t of apples and pears in 2014, only surpassed by bananas and plantains (145x10^6 t) and citrus (138x10^6 t). In these species fruit production depends to a great extent on the control of flowering. In spite of its importance, our capacity to control flowering time in fruit trees is very limited and some crucial problems, mainly alternate bearing, are far from being resolved. Hence, a clear knowledge of the genetic and molecular aspects controlling flowering is of prime interest. In addition, environmental and genetic factors associated to flowering, such as bud dormancy or chilling requirements, have yet to be addressed so as to improve fruit tree crops by either breeding or other biotechnological approaches.

Loquat [*Eriobotrya japonica* (Thunb.) Lindl.] also belonging to the family Rosaceae, subfamily Amygdaloideae, of a relative economical importance, with a production of 549x10^3 t worldwide; China is the main producer and Spain is the world's leading exporter (Lin 2007). In contrast to other pomaceous species, loquat is an evergreen species which reproductive development is a continuous process that is not interrupted by a period of winter dormancy; thus, its buds do not exhibit true dormancy and do not need chilling. Therefore, the apical meristem grows continuously developing regularly into a panicle that blooms and sets in autumn, develops the fruit during winter, and it ripens in spring (see review by Lin et al. 1999) (see Online Resource 1). Thus, loquat allows for the study of the mechanism of flowering process more accurately than other Rosaceae fruit tree species, and constitutes a strategic species to better understand how to control flowering and increase yields.

Loquat has two flushes of bud sprouting with a short rest period in between, the first one early in spring after harvest and the other in summer at full vegetative growth (Agustí and Reig 2006) (see Online Resource 1). The first flush emerges from one or two buds located at the tip of the fruiting shoot, developing current shoots (the main shoots) that first grow vegetatively (reaching 40-70 cm in length and 8-15 leaves) until the end of summer, when the apex develops an inflorescence (one panicle per shoot and 70-125 flowers per panicle). The second flush develops premature shoots (those generated as secondary shoots from the current shoots) from some of the axillary buds located in the middle of the current shoot. On average, 1 to 3 axillary buds are developed from a current shoot. These shoots also grow vegetatively in summer (up to 20-30 cm long and 8-12 leaves) and always develop an inflorescence (45-90 flowers per panicle). Interestingly, although flower bud differentiation in premature shoots is visible later than in current shoots, anthesis takes place at practically the same time in both kind of shoots (Agustí and Reig 2006). Besides, almost all shoots that developed on the tree are flowering shoots, and therefore the number of panicles per tree remains practically constant every year.

In Rosaceae woody fruit tree species, floral bud induction occurs during the vegetative period of bud development (Hanke et al. 2007). In loquat, one-year-old leaf removal assays (Fatta del Bosco 1961) revealed that flower induction takes place from early May to early June. This process is subjected to both environmental and endogenous control since flowering occurs only once a year. Fruits and leaves are responsible for endogenous control. Fruit inhibits flowering (Monselise and Goldschmidt 1982), the intensity of the inhibition depending on the species, and also, as for loquat, on the time the fruit remains on the tree (Reig et al. 2014b). Regarding leaves, two types of leaves are present on loquat trees at the time of flowering induction, old leaves generated in the previous season and young leaves emerging from current shoots. At this time, young leaves are expanding leaflets

of a few centimeters in length that need to reach a certain stage of maturity to be receptive and produce the inductive signal (Bernier et al. 1981; Batten and McConchie 1995). Since some of the axillary buds of these young leaves develop the premature shoots, which always develop into panicle, these leaves from the current shoots might also act as source of the flowering signal. This hampers the identification of which of the two, old and young leaves, or whether both, are able to perceive the flowering-inducing conditions to generate the flowering signal. This also indicates that the role in the control of flowering of these two sorts of leaves might be different.

The protein encoded by the *FLOWERING LOCUS T* (*FT*) constitutes a long-distance signal *per se* activated in leaves by species-specific stimulus, which regulate shoot meristematic activities associated to flowering, and controlling the switch between vegetative and reproductive growth (Zeevart 1976; Turck et al. 2008). FT plays a pivotal role in the flowering process in many fruit tree species, including Rosaceae woody species, such as apple (Kotoda et al. 2000; Hättasch et al. 2008), pear and quince (Esumi et al. 2005), Japanese pear (Ito et al. 2014), Japanese apricot (Esumi et al. 2009), and peach (Zhang et al. 2008), as well as other species such as citrus (Endo et al. 2005), poplar (Hsu et al. 2011), and grapevine (Sreekantan and Thomas 2006; Carmona et al. 2007). Besides flowering, FT-like proteins also regulate a wide range of developmental processes displaying either similar functions, with different spatial (tissues) or temporal expression patterns, or different functions (Pin and Nilsson 2012; Kotoda et al. 2010; Hsu et al. 2011).

The objective of this study was to identify the genes involved in bud sprouting and floral bud induction in loquat. Fisrt, it was determined when the endogenous conditions trigger bud sprouting and when the floral bud induction takes place. For the former, fruiting shoots were defruited at different fruit developmental stages to force the buds to sprout, and bud sprouting date recorded. For the latter, an exhaustive defoliation analysis and determination of flowering time were performed. Both experiments were carried out in two locations in the Mediterranean coast of Spain. The involvement of two loquat *FT*-like genes in loquat bud sprouting and flowering was studied, and analyzed their expression pattern in old leaves (one-year-old leaves) and emerging young leaves. Data suggest a role of *EjFT1* in bud sprouting and leaf development, and *EjFT2* in flowering.

Materials and methods

Plant material and location

Experiments were conducted in 2014-2016 on five- and fifteen-year-old ‘Algerie’ loquat trees [*Eriobotrya japonica* (Thumb.) Lindl.] grafted onto seedling rootstock and grown in the experimental fields at the *Universitat Politècnica de València*, Spain (39º 29’ N, 00º 20’ W) and *Cooperativa Agrícola de Callosa d’En Sarriá*, Alicante, Spain (38º 39’ N, 00º 06’ W).

Assessment of floral bud inductive period

To determine the period of floral bud induction, five-year-old ‘Algerie’ loquat trees grown in Valencia were completely and continuously defoliated during three periods: from 1 May to 25 May, from 22 May to 20 June, and from 07 June to 24 June 2014. A fourth set of trees were not defoliated and used as control trees. Four trees per

period and four control trees were used. At flowering time, number of vegetative and reproductive shoots per tree were recorded. Results are expressed as percent of reproductive shoots per tree.

Assessment of *FT* expression, flowering, and bud sprouting

To study the time-course of *FT* expression during floral bud inductive period, one-year-old leaf samples (old leaves) and emerging new leaf samples of current shoots (young leaves) were collected from early May to early July 2014 from three trees grown in Valencia and Callosa, and four trees grown in Valencia, respectively. Young leaves were classified and labelled as G1 to G6 generation (G) as they were emerging (the oldest G1 is the first to emerge on the shoot), and collected first those from G1 generation, second those from G1 and the new G2 generation, third those from G1, G2 and the new G3 generation, and so on up to G6 generation, as shown in Online Resource 2.

To study the *FT* expression and bud sprouting relationship, three shoots per tree of four trees grown in Valencia were defruited to force bud sprouting at fruit set (02 January, D1), early fruit development (10 February, D2), fruit colour break (20 March, D3), and at harvest (01 April, control). Old leaves were sampled during the 30-60 days following the defruiting, and date of bud sprouting was recorded.

In the two experiments, three samples per date were collected at 11-12 a.m. to minimize circadian or light effects in gene regulation, and immediately frozen in liquid N_2 and stored at -80ºC until RNA extraction.

cDNA amplification by 5' and 3' RACE

RACE was performed using the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) and Advantage 2 Taq Polymerase (Clontech, Mountain View, CA, USA). Total RNA from leaves was extracted according to Martínez-Fuentes et al. (2015). RNA quality was tested by 260/280 absorbance 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. Oligos were designed at the most conserved regions of the Coding DNA Sequence (CDS) of *MdFT1* (Genebank AB458506), *MdFT2* (AB458505), *PpFT1* (AB524587) and *PpFT2* (AB571595) from the Phytozome database (MDP0000139278 and MDP0000132050) (Online Resource 3). All RACE products were cloned in the pGEM-T-Easy vector (Promega, Madison, WI, USA) and fully sequenced from both ends using vector primers.

Genomic DNA amplification

Genomic DNA was extracted from 200 mg of young loquat leaves following the method described by Doyle and Doyle (1987) with modifications (Gisbert et al. 2009). To amplify *FT* genes from genomic DNA, oligos were designed at the conserved region in the 5´ and 3' untranslated regions (UTRs) of *EjFT2* cDNA isolated by RACE (see Results) and *MdFT1* and *MdFT2* genomic sequences (Online Resource 3). PCR was carried out from genomic DNA in 30 cycles of 30 s at 94°C, 30 s at 55°C, and 4 min at 68°C, followed by an extra extension of 10 min at 68°C, using Accuzyme™ DNA Polymerase (Bioline, Tauton, MA, USA). PCR products were cloned in pGEM-T-Easy vector (Promega, Madison, WI, USA) and sequenced.

Sequence analysis

Sequences were aligned using the UGENE software with ClustalW (Okonechnikov et al. 2012). For the phylogenetic analysis, protein sequences were aligned with the MEGA software with ClustalW algorithm (Tamura et al. 2013) using maximum likelihood method and bootstrapped with 1000 iterations.

Expression analysis by qPCR

RNA isolation, quantification, and quality analysis was performed as described above (Martínez-Fuentes et al. 2015). Quantitative real-time PCR (qPCR) was performed with a LightCycler 2.0 Instrument (Roche Diagnostics, Basel, Switzerland) equipped with LightCycler Software version 4.0. One-step qPCR was carried out in triplicate (three technical replicas) for each biological sample. Each reaction contained 2.5 u of MultiScribe Reverse Transcriptase (Applied Biosystems, Carlsbad, CA, USA), 1 u of RNase Inhibitor (Applied Biosystems, Carlsbad, CA, USA), 2 μl LC FastStart DNA MasterPLUSSYBR Green I (Roche Diagnostics, Basel, Switzerland), 25 ng total RNA, and 250 nM of the specific forward and reverse primers of each gene, in a total volume of 10 μl. Incubations were carried out at 48ºC for 30 min and 95ºC for 10 min, followed by 45 cycles at 95ºC for 2 s, 58ºC for 8 s and 72ºC for 8 s. Fluorescent intensity data were acquired during the 72ºC-extension step and transformed to relative mRNA values using a ten-fold dilution series of RNA samples as the standard curve. Relative mRNA levels were then normalized to total mRNA amounts (Bustin 2002). In each case, an expression value of 1 was arbitrarily assigned to the sample collected at the first time point (the earliest date). *ß-actin* was used as the reference gene (Shan et al. 2008). Specificity of the amplification reactions was assessed using post-amplification dissociation curves.

For the preliminary qPCR analysis, oligos were designed using the conserved regions of *FT*s from the Rosaceae family, specifically those *Pyrus pyrifolia* Nakai and *Malus domestica* Borkh (Online Resource 3). For the gene-specific qPCR analysis, primers were designed at the most divergence regions of the cDNAs of *EjFT1* and *EjFT2* to avoid cross amplification (Online Resource 3). Oligos were cross-tested performing PCR using as template *EjFT1* and *EjFT2* cDNAs cloned in pGEM-T-Easy (Online Resource 4).

Results

Determination of the floral bud inductive period

To establish the floral bud inductive period in loquat and to determine the effects in flowering, an exhaustive defoliation analysis was carried out in spring-summer 2014. When all leaves (old and young leaves) were continuously removed from 22 May to 20 June or from 7 June to 24 June no flowers were developed in autumn. In contrast, when leaves were continuously removed from 1 to 25 May 100% of shoots developed into panicles as in not defoliated control trees, (Fig. 1). Accordingly, from early (7) to late (20)-June the presence of leaves was necessary for the apex to develop into a panicle, whereas it was not necessary from early-May (1 May) to early-June (7 June) in our experiment, and from late-June (20 June) onwards. This allowed us to establish mid-June

(from 7 to 20 June in our 2014 experiment in the Northern Hemisphere) as the floral bud inductive period in loquat.

Expression of *FT*-like genes in leaves

Both old and young leaves seem to be involved in the inductive floral process in loquat. To identify the role of the known flowering genes in loquat, the temporal expression pattern of the orthologous *FT* gene was studied using oligos designed from a conserved region of *FTs* from closely related species (*Pyrus pyrifolia* Nakai and *Malus domestica* Borkh). To this end, old leaves were collected from 15 d after bud sprouting until early in summer, and young leaves as they emerged from current shoots throughout the spring and summer, i.e. previous to, during, and until one month after the floral bud inductive period detected, grouping them into six generations (G1 to G6, being G1 the oldest and G6 the youngest). The time-course of relative expression of *FT* in old leaves exhibited two peaks of expression, around mid-May and mid-June (Fig. 2), the latter coinciding with the period of floral bud induction (Fig. 1) found in the trees grown in the two locations, but not the former. This one coincided with the vegetative growth of the new shoots sprouted after harvest. It is noteworthy that regardless of the location, during the time-course of relative expression of *FT* in old leaves, young leaves and old leaves coexisted on the tree, and therefore both might be involved in the inductive floral process in loquat. In young leaves, a peak of *FT* expression was also observed in mid-June (15 June) for generations G1 to G4, while for G5 and G6 it was almost imperceptible (Fig. 3). Interestingly, the high relative expression of *FT* in the older leaves (generations G1 and G2) around early May correlated with the emergence of leaves. Lastly, leaves from generations G5 and G6 did not display substantial up-regulation at the time of floral bud inductive period (15 June in our experiment) (Fig. 3), indicating that only leaves emerged at least 15 d earlier peaked at this time.

On the other hand, the two peaks of *FT* expression found in old leaves (Fig. 2), as well as the presence of two *FT* genes in *Arabidopsis, Populus, Malus and Citrus*, suggested that there are probably more than just one *FT*-like gene in the genome of loquat. The isolation and functional analysis of such genes were required to test gene specific expression profiles and for a better understanding of the molecular mechanisms of flowering in loquat.

Isolation of *FT1* and *FT2* loquat genes

To identify the *FT* homologues in loquat, rapid amplification of cDNA ends (RACE) was used to generate cDNA fragments from RNA from old, fully-expanded leaves during the floral bud inductive period. RACE primers were designed based on the sequences of *FT1* and *FT2* from *Pyrus pyrifolia* and *Malus domestica*. The 5´ and 3´ RACE fragments were 272 and 472 bp long, respectively. While the 3´RACE fragment covered the whole putative coding sequence, the 5´fragment did not contain the ATG start codon; therefore, a new 5´RACE primer to isolate a 391 bp product that covered the putative 5' end cDNA was designed. All RACE products isolated corresponded with a single gene. Using the sequence from these three fragments, two new primers at both ends of the putative cDNA were designed to amplify a complete cDNA sequence. The full length cDNA was 1057 bp-long and encoded a putative protein of 174 aa (Online Resource 5A). Basic local alignment search tool (BLAST) analysis revealed

that the corresponding gene was quite similar to *Pyrus* and *Malus FT2*; therefore, the new isolated cDNA from loquat was named as *EjFT2* (GeneBank KP941772).

As all RACE products corresponded to the *EjFT2*, a putative *EjFT1* from genomic DNA was amplified using oligos designed from the genomic sequence of *FT1* from *Malus*. Two PCR products of 2684 bp and 2905 long that corresponded to two different alleles of a single gene that differed in a 163-bp fragment in the third intron were obtained (Online Resource 6) (Genebank KP941773 and KP941774). The deduced cDNA encoded a 174 aa protein (Online Resource 5B) closely related to *Pyrus* and *Malus FT1*, and was named *EjFT1* (GeneBank KP941771).

Deduced proteins from *EjFT1* and *EjFT2* were highly conserved with FTs from *Malus*, *Pyrus*, *Populus* and *Arabidopsis* (Fig. 4A), particularly in the Amygdaloideae *Malus* and *Pyrus* species with percentages of similarities ranging between 99% (for *FT2*) and 100% (for *FT1*) (Online Resource 7). Phylogenetic analysis using protein sequences further showed that *EjFT1* and *EjFT2* are grouped in the same clade with the Amygdaloideae *FT* genes. Also, the phylogenetic tree situated *EjFT* genes grouped with others angiosperms *FT* and separated from the *TERMINAL FLOWER 1* (*TFL1)* and *MOTHER OF FT AND TFL1* (*MFT*) subfamilies of the common angiosperm phosphatidylethanolamine-binding protein gene family (PEBP) group. All this data suggest that these genes from loquat encode *bona fine* FT proteins (Fig. 4B).

Gene expression profile of the *EjFT1* and *EjFT2* genes and its role in bud sprouting and leaf development

Using gene-specific primers qPCR revealed a strong increase in *EjFT1* expression in old leaves during early-mid May, coinciding with young vegetative shoots development, followed by a return to initial levels by the beginning of June (Fig. 5A). By comparison, *EjFT2* mRNA followed similar kinetics in May, but not in June, peaking again during the floral bud inductive period, whereas expression of *EjFT1* did not (Fig. 5B).

A parallel examination of *EjFT1* and *EjFT2* accumulation in young leaves revealed that while *EjFT2* transcript levels remained low during May regardless of leaf generation, *EjFT1* transcriptions in generations G1 and G2 was high in mid-May, as indicated for total *FT* expression (see Fig. 3), and coinciding with that in old leaves (Fig. 5A), whereas that in G3 was delayed until the end of May (Fig. 5C). Furthermore, *EjFT1* expression was substantially higher and delayed in the youngest leaf generations (G4 and G5) until mid-June and the end of June, respectively, as compared to the first leaf generations emerged (Fig. 5C). Interestingly, the increase in *EjFT1* mRNA of the young leaf generations correlated with the time they emerged (see Online Resource 1)

Notwithstanding, *EjFT2* expression in young leaves of generations G1, G2 and G3 was greatest in mid-June, i.e. at floral bud inductive period, irrespective of when they emerged and coinciding with the second peak of *EjFT2* expression found in old leaves (Fig. 5B), whereas expression in leaves of the youngest G4 and G5 generations remained low (Fig. 5D).

Accordingly, *EjFT1* transcripts in young leaves generations exhibited a high expression level during leaf expansion, whereas those of *EjFT2* were always abundant around mid-June, but only in leaves fully expanded (G1 to G3), suggesting *EjFT1* might regulate leaf emergence/expansion while *EjFT2* would be involved in floral bud induction.

In order to assess whether *EjFT1* expression patterns might help define the young leaf flush initiation/expansion and to correlate between *EjFT1* expression and young leaves' onset, we examined *EjFT1* and

EjFT2 expression in old leaves after fruit removal, since fruit removal forces bud sprouting and, thus, young leaf development. Shoots were defruited at different fruit developmental stages, i.e. at fruit set (D1), early fruit growth (D2), fruit colour break (D3), and fully fruit maturity (control). Fruit removal advanced *EjFT1* expression in old leaves regardless of the time the fruit remained on the tree in reference to harvest date (1 April). Furthermore, the sooner the fruit detached, the sooner the bud sprouted (Fig. 6A). In all cases qPCR analysis in old leaf exhibited a peak of *EjFT1* expression 22-28 d after defruiting and 8-12 d before new buds sprouted, the young leaves emerging 30-40 d after fruit removal (Fig. 6A). This suggests that *EjFT1* might be responsible for leaf emergence in loquat. However, *EjFT2* expression peaked 20-50 d after defruiting and it did not correlate with young leaf flush initiation (Fig. 6B).

Discussion

In loquat, when all leaves of the tree were continuously removed from early- to late-June vegetative apex did not differentiate into panicles. This assay allowed us to determine the floral bud inductive period in loquat at mid-June. This period is also in agreement with our previous studies into the effect of gibberellic acid in reducing flowering intensity in loquat (Reig et al. 2011). Fatta del Bosco (1961) established that the floral bud inductive period of loquat starts in early May, but the author only defoliated one branch per tree and only used two three-years-old trees per data, making the results imprecise. Interestingly, mid-June is also the floral bud inductive period for apple (Kotoda et al. 2000), despite the differences in flowering and development observed in apple and loquat.

In woody perennials, shoot meristem exhibits transitions between vegetative and reproductive development. In some species, these vegetative-reproductive cycles are harmonized by transitory expression of *FT1* and *FT2* paralogs, which diverge in function and temporal expression (Pin et al. 2010; Hsu et al. 2011). Given that in our experiment the mRNA level of total *FT* began to increase early in May, peaking around the second ten of May, and the process re-started one month later, peaking in mid-June, it seemed that a similar scenario for the two *FT* genes occurs in loquat. Moreover, as the *Malus* and *Pyrus* genomes encode for two *FT* genes (Kotoda et al. 2010; Ito et al. 2014), it was expected that loquat, a close relative in the subfamily Amygdaloideae, might also have two *FT* paralogs. Sequence analysis of the two genes isolated from loquat reveals that one of them was 100% and 98% similar to *MdFT1* and *PpFT1a* (Online Resource 7), respectively, and was named *EjFT1*, whereas the percentage of similarities for the second one was 99% to both *MdFT2* and *PpFT2a* (Online Resource 7), and was named *EjFT2*. Since all known Amygdaloideae species come from a common ancestor (Evans and Campbell 2002) and they all would therefore present two *FT* gene copies, the most parsimonious scenario is that duplication of *FT* genes was simultaneous or previous to the origin of Amygdaloideae. Several members of the *FT/TFL1* (flowering-promoter and the repressor) family have been identified in *Arabidopsis* (Shannon and Meeks-Wagner 1991), citrus (Nishikawa et al. 2007), grapevine (Carmona et al. 2007), *Poplar* spp. (Igasaki et al. 2008; Hsu et al. 2011), apple (Kotoda et al. 2010), and peach (Chen et al. 2013).

In perennial poplar, Hsu et al. (2011) reported that *FT1* and *FT2* homologues of *Arabidopsis* coordinate vegetative and reproductive growth. In apple, Kotoda et al. (2010) isolated two paralogs of *FT*, *MdFT1* and *MdFT2*, both having the potential to induce flowering when expressed in *Arabidopsis*. Since *EjFT1* and *EjFT2* have highly conserved sequences in each clade from apple and other Rosaceae species (Online Resource 7), it was

assumed that they are the functional *bona fine FT* genes in loquat. In any case, their expression in old and in continuous generations of young leaves was analyzed to validate these assumptions. In old leaves *EjFT1* was expressed in the middle of May whereas *EjFT2* did so in the same period but also in the middle of June. *EjFT1* also peaked 10 d, approximately, before bud sprouting forced by defruiting shoots and irrespective to the year season, whereas *EjFT2* did not correlate. However, the pattern of expression for young leaves depended on the age of leaf, *EjFT1* peaking during leaf expansion on all sample dates (i.e. to leaflet generation, G), and *EjFT2* peaking in the middle of June irrespective to leaf generation, although certain stage of maturity to allow gene expression was needed, as reported for other species such as lychee (Batten and Mc Conchie 1995), macadamia (Olesen 2005), and tomato (Shalit et al. 2009). In fact, in many species flowering is generally accompanied by an increase in leaf growth rate (Bernier et al. 1981). Thus, age-dependent gene expression gradients were patent in leaflets of the sequentially vegetative flushes (leaf generations) along the current shoots in loquat. *EjFT1* and *EjFT2* display differences in leaf age-dependent expression gradient in which *EjFT2* expression was relatively high in expanded mature leaves and *EjFT1* in the immature leaves.

In our experiments, any differences in terms of the pattern and levels of expression of *EjFT1* and *EjFT2* in old leaves in the middle of May failed to be found. Loquat sprouts at this time, immediately after fruit harvest, and therefore, both *EjFT1* and *EjFT2* might control vegetative growth in accordance with the pleiotropic function of *FT*-like genes, i.e. with the multifaceted role of duplicated FT-proteins (Pin and Nilsson 2012). The increasing expression in *EjFT1* before bud sprouting and during young leaf expansion suggests that vegetative growth and leaf expansion may be controlled by this gene (see Fig. 7). Further, *EjFT2* expression in young leaves occurs when they become mature leaves, just when *EjFT1* is suppressed, indicating that *EjFT1* regulates leaf growth. However, our results, by removing the fruits, found no correlation between the expression of *EjFT2* and the onset of young leaves. Notwithstanding, the increase in its expression in the middle of June might be important for transition from the vegetative to the reproductive development. The expression pattern of *EjFT2* in old and young leaves, coinciding with the floral bud inductive period, indicates that it is one of the candidates for flowering induction. A schematic representation showing how *EjFT1* and *EjFT2* regulate bud sprouting and floral bud induction is presented in Fig. 7.

The spatial and temporal expression patterns of both *EjFT1* and *EjFT2* were determined by conducting an extensive period transcript analysis before, during, and after floral bud inductive period established by leaf removal. In this regard, it is essential to highlight that the presence of growing fruits prevents buds from sprouting, and sprouting is delayed as long as fruit remained on the tree (Reig et al. 2014*a*). This is in accordance with the *EjFT1* and *EjFT2* expression patterns and suggests that persisting fruit might prevent their expression. In fact, when fruits were removed from shoots *EjFT1* expression pattern peaked 22-28 d later and *EjFT2* 20-50 d later, whereas in not-defruited shoots no expression was found. Fruit developmental stage at defruiting explains the differences in *EjFT2* expression date, so that the latter defruiting the sooner the peak of expression. Taken together, these results, as well as those found in other species (Tamaki et al. 2007; Shalit et al. 2009; Hsu et al. 2011; Hiraoka et al. 2013), indicate that axillary buds of loquat are highly sensitive to overexpression of *EjFT1* and, thus, lateral shoot outgrowth and its subsequent elongation seem to be an integral part of the *FT* gene actions.

It explains the ability of loquat to develop premature shoots in summer that also develop into panicles. Most of the buds generating these secondary shoots did not exist at the time of floral bud induction and, nor did their subtending leaves (Reig and Agustí 2011), but the apical bud of the current shoot did exist. The abundance of

EjFT2 transcripts during rapid shoot growth also in summer (data not shown) might explain the relatively high proportion of premature shoots developing into panicle in loquat.

In summary, two *FT* genes, *EjFT1* and *EjFT2*, were isolated and sequenced from leaves of *Eriobotrya japonica* and identified their expression during vegetative growth and floral bud induction. Combined gene expression patterns and defoliation and defruiting assays strongly suggest that *EjFT1* is related to bud sprouting and leaf development, while *EjFT2* is involved in floral bud induction. Our studies showed that transcription of *EjFT1*, which peaked together with that of *EjFT2*, is upregulated one month before flower induction and is reduced just before it, its suppression allowing for the accumulation of *EjFT2* transcripts resulting in floral bud induction. This time frame is equivalent to the first half of June, coinciding with the second peak of *EjFT2* expression, suggesting that this gene regulates flower induction 2-3 months before the first visible morphological changes in the apical meristem occurs. This peak of expression appears in one-year-old leaves and also in current young leaves for which a certain stage of maturity is required to become receptive and produce the flowering induction signal.

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Legends to Figures

Fig. 1 Influence of leaf removal on the percentage of flowering shoots in 'Algerie' loquat trees grown in Valencia, Spain. Black boxes indicate the period for which the tree lacked leaves. Blank boxes indicate no leaf removal. Values are from 6 five-year-old trees

Fig. 2 Relative expression pattern of *FT* in old leaves of 'Alerie' loquat. Trees were grown in two locations, Callosa (Alicante) (A) and Valencia (B), Spain. Values are means ± SE of three replicates. In some cases SE is smaller than the symbol size

Fig. 3 Relative expression pattern of *FT* in leaves of six consecutive generations (G1 to G6) of young leaves of 'Algerie' loquat trees grown in Callosa (Alicante, Spain). Values are means ± SE of three replicates. In some cases SE is smaller than the symbol size

Fig. 4 Sequence analysis of the deduced FT proteins from 'Algerie' loquat and other FT proteins using ClustalW algorithm (A) and representation of the phylogenetic relationships using a Maximum Likelihood phylogenetic tree (B). Numbers next to each node represents the bootstrap values. The scale bar indicates the branch length that corresponds to the number of substitutions per aminoacid position. Accesion IDs: EjFT1, KP941771; EjFT2, KP941772; MdFT1, AB458506.1; MdFT2, AB458504.1; PpFT1a, AB524587.3; PpFT2a, AB571595.3; PtFT1, POPTR_0008s07730.1; PtFT2, POPTR_0010s18680.1; AtFT, AB027504.1; AtTSF, AB027506.1; AtMFT, NM_101672.3; AtTFL1, NM_120465.2. Aminoacids with consensus higher than 80% are shown in dark grey, those with 60%-80% consensus in grey, those with 40%-60% in pale grey, and those with consensus lower than 40% in white
Ej, *Eriobotrya japonica*; Md, *Malus domestica*; Pp, *Pyrus pyrifolia*; Pt, *Populus trichocarpa*; At, *Arabidopsis thaliana*

Fig. 5 Expression pattern of *EjFT1* (A and C) and *EjFT2* (B and D) in old leaves (A and B), and in young leaves (C and D) of six consecutive generations (G1 to G6) of 'Algerie' loquat trees grown in Callosa (Alicante, Spain). Values are means ± SE of three replicates. In some cases SE is smaller than the symbol size

Fig 6. Expression pattern of *EjFT1* (A) and *EjFT2* (B) in old leaves of shoots defruited at fruit set (D1), early fruit growth (D2), fruit colour break (D3), and at harvest time (Control) of 'Algerie' loquat trees grown in Valencia (Spain). Values are means ± SE of three replicates. The arrows indicate the first leaf primordia emerging date for each defruiting date. In most cases SE is smaller than the symbol size

Fig 7. Schematic representation showing how *EjFT1* and *EjFT2* may regulate bud sprouting and floral bud induction in loquat. In old leaves (solid areas), both genes are downregulated by developing fruit and their expression occurs after harvest. *EjFT1* induces bud sprouting. A second peak of expression of *EjFT2* occurs in coincidence with floral bud inductive period, and induces flowering. In young leaves (empty areas), *EjFT1* expresses before a new generation appears, and *EjFT2* expresses later in summer (dashed line indicates

missing samples). In young leaves, *EjFT2* expression occurs once the *EjFT1* is suppressed. In summer, a premature shoots development occurs. Buds from which they developed and their subtending leaves did not exist at the floral bud inductive period, however premature shoot regularly developed into panicle in autumn at the same time that current shoot did it. Expression of *EjFT2* in summer might explain the development into panicle of premature shoots