Plant Science 335 (2023) 111810

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Warm temperature during floral bud transition turns off *EjTFL1* gene expression and promotes flowering in Loquat (*Eriobotrya japonica* Lindl.)

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ARTICLE INFO

Keywords:
Loquat
Summer-heat dormancy
Floral bud differentiation
EjTFL1
EjAP1
EjLFY
ABA

ABSTRACT

The Rosaceae family includes several deciduous woody species whose flower development extends over two consecutive growing seasons with a winter dormant period in between. Loquat (*Eriobotrya japonica* Lindl.) belongs to this family, but it is an evergreen species whose flower bud initiation and flowering occur within the same growing year. Vegetative growth dominates from spring to late summer when terminal buds bloom as panicles. Thus, its floral buds do not undergo winter dormancy until flowering, but a summer heat period of dormancy is required for floral bud differentiation, and that is why we used loquat to study the mechanism by which this summer rest period contributes to floral differentiation of Rosaceae species. As for the deciduous species, the bud transition to the generative stage is initiated by the floral integrator genes. There is evidence that combinations of environmental signals and internal cues (plant hormones) control the expression of *TFL1*, but the mechanism by which this gene regulates its expression in loquat needs to be clarified for a better understanding of its floral initiation and seasonal growth cycles. Under high temperatures (>25ºC) after floral bud inductive period, *EjTFL1* expression decreases during meristem transition to the reproductive stage, and the promoters of flowering (*EjAP1* and *EjLFY*) increase, indicating that the floral bud differentiation is affected by high temperatures. Monitoring the apical meristem of loquat in June-August of two consecutive years under ambient and thermal controlled conditions showed that under lower temperatures (<25ºC) during the same period, shoot apex did not stop growing and a higher *EjTFL1* expression was recorded, preventing the bud to flower. Likewise, temperature directly affects ABA content in the meristem paralleling *EjTFL1* expression, suggesting signaling cascades could converge to refine the expression of *EjTFL1* under specific conditions (T^a<25ºC) during the floral transition stage.

1. Introduction

The Rosaceae family includes several economically important deciduous species such as apple, pear, peach and plum whose flower development extends over two consecutive growing seasons (Kurokura et al., 2013). During the first season, flower induction, initiation and organ differentiation take place in summer, and most shoot meristems change to reproductive growth and initiate flowers. After winter dormancy, flower development continues during the second season and ends with anthesis in the spring (Hanke et al., 2007). Loquat (*Eriobotrya japonica* Lindl.) belongs to this family, but it is an evergreen species whose flower bud initiation and flowering occur within the same year and whose flower buds do not undergo dormancy until flowering (Jiang et al., 2019). Vegetative growth dominates from spring to summer, when the floral transition begins in the terminal buds of newly developed vegetative shoots; then, after flower initiation in late summer, these terminal buds bloom as panicles, so that the process of flower initiation to flowering is completed within a few months, as in some recurrent flowering Rosaceae, such as *Rosa* sp. and *Fragaria vesca* (Bendahmane et al., 2013).

Research into flowering pathways mainly focuses on the annual model *Arabidopsis thaliana* and also the perennial *Arabis alpine* (Wang et al., 2009), which is only barely comparable with temperate fruit trees like loquat. Perennial plant species differ greatly from annuals in their life cycle and physiological requirements, and therefore might differ in the molecular mechanisms that control flowering (Jung and Müller, 2009). In any case, the time of floral initiation is governed by floral promoters and repressors that respond to environmental and endogenous cues. In *Arabidopsis*, the transition to the generative stage is initiated by four independent flowering pathways, photoperiod,

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https://doi.org/10.1016/j.plantsci.2023.111810
Received 2 May 2023; Received in revised form 19 July 2023; Accepted 21 July 2023
Available online 25 July 2023
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vernalization, gibberellin-dependent and autonomous. Photoperiodic and temperature changes mediate the expression of floral integrator genes like *FLOWERING LOCUS T* (*FT*), *CONSTANS* (*CO*) and *FLOWERING LOCUS C* (*FLC*) in leaves, and *FLOWERING LOCUS D (FD), SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), *LEAFY* (*LFY*), *APETALA1* (*AP1*) and *TERMINAL FLOWER 1* (*TFL1*) in the shoot apical meristem (SAM) (Ratcliffe and Riechmann, 2002). In woody Rosaceae species, such as apple, the genome contains several candidate genes homologous to *Arabidopsis* vernalization and ambient temperature pathway genes, while two *MdFLC*-like genes have been found in leaves (Guitton et al., 2012; Kumar et al., 2016). However, in the meristem, the *TFL1* gene was identified as a key regulator, which represses flowering and maintains the vegetative state of the meristem (Ratcliffe and Riechmann, 2002). FLC when expressed in leaves represses *FT*, while *TFL1* is expressed in the shoot apical and axillary meristems and inhibits the activity of *AP1* and *LFY* at the center of the shoot apex by delaying their up-regulation and, thus, preventing the meristem from responding to *LFY* or *AP1*. In turn, the *FT*-promoted genes *LFY*, *AP1* and *CAULIFLOWER* (*CAL*) prevent *TFL1* transcription in floral meristems on the apex periphery, allowing for indeterminate inflorescences to form (Hanano and Goto, 2011).

In apple, the homologous gene to *TFL1* (*MdTFL1*) is involved in maintaining the vegetative meristem identity, acting redundantly as a floral repressor (Mimida et al., 2009). In three rosaceous species tested so far, the expression maxima of *TFL1* is detected in the vegetative SAM whereas the expression levels of *MdAP1*, *AFL* (*LFY* homologue) and *MdFT* are up-regulated in the terminal shoot apex (Hättasch et al., 2008; Mimida et al., 2011b). As the SAM ceases to produce new primordia, shoot growth stops, and floral differentiation begins.

The use of *Arabidopsis* as a model system has elucidated the role of new players in the temperature-signalling network in plants. Apart from vernalization responses, ambient temperature regulates flowering by two genetic pathways: one that is closely associated with the photoperiod pathway and requires *ELF3*, and another that requires *TFL1* and is related to the autonomous pathway (Strasser et al., 2009). Hence, the role of the identified ambient temperature-sensing mechanism (Kumar et al., 2012) should be analyzed in relation to *TFL1* expression, which may control temperature-dependent flowering.

On the other hand, modulation of hormone signalling also contributes to the plasticity of the flowering process. Interestingly, different hormone signalling cascades often converge to refine the expression of key floral genes under specific conditions. In addition to these well-established phytohormones, several diffusible molecules including sugars and other metabolites regulate flowering (Wahl et al., 2013). Studies in apple and pear indicate that hormone balance may control flowering in these species. Bending the lateral branch to a horizontal position can increase the number of flower buds per shoot (Han et al., 2007) by inducing high cytokinin/auxin ratios in the lateral buds (Banno et al., 1985). Recently, the analysis of transgenic apple containing an *MdTFL1* promoter:GUS *(β-glucuronidase)* construct showed that these two hormones can induce the expression of *MdTFL1* in shoot apices (Mimida et al., 2011a). Expression of *MdTFL1* is enhaced by combining cytokinin and auxin in shoot apices, thus determining whether the shoot apical meristem will transit from the vegetative to reproductive phase (Mimida et al., 2011b). Appliying exogenous gibberellin (GA) has been shown to inhibit flowering in many perennial fruit trees such as apple (Tromp, 1982), *Citrus* (Monselise and Halevy, 1964) or avocado (Salazar-García and Lovatt, 2000). One possibility is that GA inhibits flowering through *TFL1*-like genes (Roberts et al., 1999); however, Mimida et al. (2011a) were unable to activate *MdTFL1* through in vitro applications.

The contribution of ABA signalling to the floral transition is still controversial, as both positive and negative roles of ABA have been reported (Conti et al., 2014). Several studies offer insight on the molecular level into this negative role of ABA in flowering by showing that ABA activates *FLC* directly through the bZIP transcriptional factor *ABSCISIC ACID-INSENSITIVE 5* (*ABI5*) and the AP2/ERF domain-containig transcription factor *ABSCISIC ACID-INSENSITIVE 4* (*ABI4*) in *Arabidopsis* (Shu et al., 2016; Wang et al., 2013).

Accordingly, there is evidence that combinations of environmental signals and internal cues (plant hormones) control the expression of *TFL1*, but the mechanism by which this gene regulates its expression in loquat needs to be clarified for a better understanding of floral initiation and seasonal growth cycles.

In this study, we provide insight into the sensitivity of *EjTFL1* to warm temperatures decreasing the expression as the meristem transites to the reproductive stage promoting flowering, and showing that floral bud differentiation is highly temperature dependent. How temperature affects ABA content in the meristem paralleling *EjTFL1* expression is also examined.

2. Material and methods

2.1. Plant material, growth conditions and sampling for histological, hormonal and molecular analyses

Experiments were carried out during two consecutive summers (2019 and 2020), on six-year-old 'Algerie' loquat trees *Eriobotrya japonica* (Lindl.) grafted onto seedling rootstock and grown in the field and in a greenhouse with controlled temperatures ranging 24–26/18–21 °C (day/night), in the Universitat Politècnica de València, Spain (39º 29′ N, 00º 20′ W). Trees similar in size, vigor and potential crop load were grown in 20 L-pots, under the same conditions of soil and irrigation. Different trees were used each year. Fertilization, pest management, thinning, and pruning were in accordance with normal commercial practices.

In both years, the maximum temperature in the greenhouse did not exceed 25 °C (Fig. 1B), the relative humidity was 80% and the photoperiod was adjusted to that of the field, from 6:00 am to 22:00 pm, approximately, and radiation did not exceed 150 watts per square meter.

In the field the ambient temperature during the two summers ranged 30–40/20–25 °C (day/night), being for the first year of the experiment as shown in Fig. 1A and the average relative humidity was 66% for both summers.

In 2019, twenty-four trees were selected for the experiment and were grown under field conditions until the 25 June when twelve of them were moved to the greenhouse until the 28 August.

In 2020, thirty-six trees grown in the field were separated into six groups of six trees each. On the 25 June three groups of trees were placed in a greenhouse until the 15 July, 27 July and 24 August, when one of each were moved to the field. The other two groups remained in the field until the 15 and 27 July, respectively, when they were moved to the greenhouse. Finally, another group remained in the field throughout the experiment as a control (Fig. 2).

For hormonal and molecular analyses, four apical meristems per tree of three trees were sampled periodically from late June to late August, according to their developmental stage, during the first summer of the experiment.

In the second summer, four apical meristems per tree of three trees per group were sampled during mid-late July (15th and 27th) and during mid-late August (11th and 24th). In both cases, three technical replicates per date were collected and immediately frozen in liquid N_2 and stored at − 80ºC for RNA extractions and RT-PCR analysis and for hormonal analyses.

Morphological changes in the apical meristem were monitored during the floral transition, from early to end August, with a photographic camera (E600 NIKON digital) attached to the microscope and processed using a Quantiment 570 Image Analysis System (Leica Cambridge). One apical bud was sampled per tree and date of 6 trees.

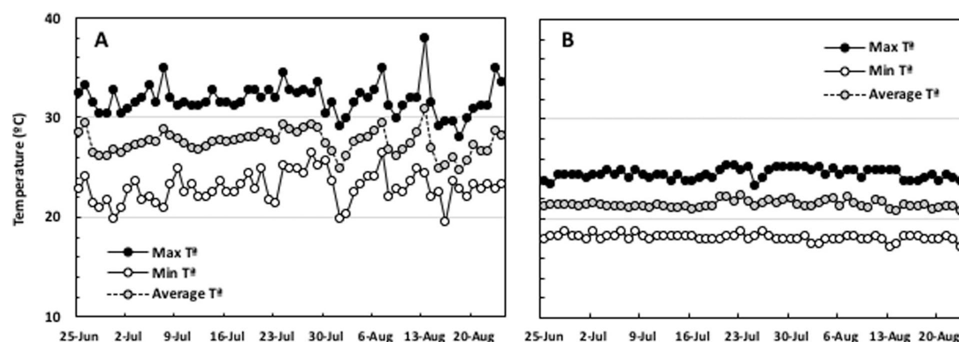

Fig. 1. Maximum, minimum, and average temperature in the field (A) and indoors (B) from June to August during the first summer of the experiment.

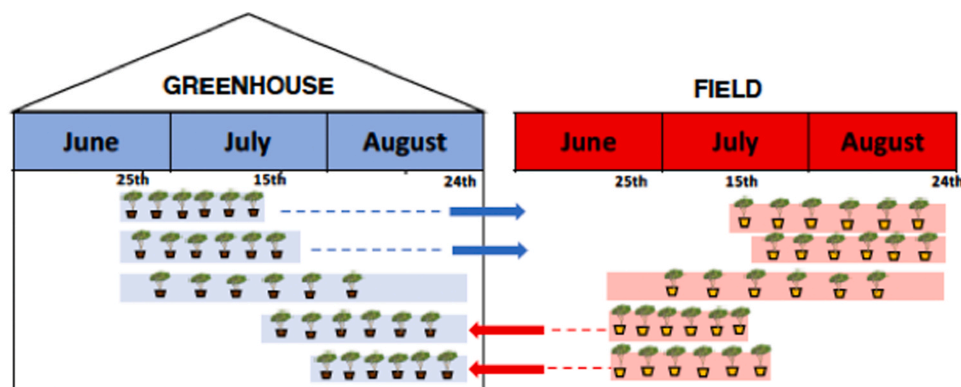

Fig. 2. Periods under indoor and field conditions for each group of trees during the second summer of the experiment.

2.2. RNA extraction and gene expression analysis by RT-PCR

RNA isolation, quantification, and quality analysis were performed as described by 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 µl of MultiScribe Reverse Transcriptase (Applied Biosystems, Carlsbad, CA, USA), 1 µl of RNase Inhibitor (Applied Biosystems), 2 µl LC FastStart DNA MasterPLUSSYBR Green I (Roche Diagnostics), 25 ng of 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 48 °C for 30 min and 95 °C for 10 min, followed by 45 cycles at 95 °C for 2 s, 58 °C for 8 s and 72 °C for 8 s. Fluorescent intensity data were acquired during the 72 °C-extension step and transformed to relative mRNA values using a tenfold 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). *β-actin* was used as the reference gene (Shan et al., 2008). Specificity of the amplification reactions was assessed using post-amplification dissociation curves.

The relative expression of RNA transcripts was quantified with the threshold cycle values (Ct) obtained from each sample using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The relative gene expression level is given by $2^{-\Delta\Delta Ct}$. Normalization was performed to the first sample date for each species. Three independent biological samples under each experimental condition were evaluated in technical triplicates.

For the qPCR gene analysis, we considered the following oligos: *EjAP1, EjLFY, EjTFL1* (Liu et al., 2013; Esumi et al., 2005).

The primers used for qRT-PCR analysis are listed in Table 1.

Table 1
Primer sequence used in RT-PCR amplification reactions.

Annotation	NCBI accession number	5′ -Direct primer- 3′ 5′-Reverse primer- 3′	Predicted product (bp)
ACTIN	JX089586	ATGAGGGAGGGCATAACC TGTTGCCATACAGGCTGT	**121**
EjLFY	AB162033	ATCCAGGTCCAGAACATTGC ATGTAGCTTGCGCCTGACTT	**100**
EjAP1	AY880262	AGCTGGACCTGACTCTGGAA TGATGATCAAGCAGCAAAGC	**65**
EjTFL1	GU320722	TCTGTTGTCACAGCCAAACC AGTGCAGGTGCTCCCTTAGA	**65**

2.3. Plant hormone analyses

Aliquots (about 50 mg DW) of ground material were extracted with 80%methanol containing 1% acetic acid. Internal standards were added and mixed with the aliquots at 4 °C for 1 h. Deuterium-labelled hormones were used as internal standards for plant hormone quantification.

The extraction protocol was carried out according to Seo et al. (2011) with some modifications. In brief, for desaltation, the extracts were passed through reverse phase columns HLB (Waters Cromatografía,S.A., Barcelona, Spain). The plant hormones were eluted by 80% methanol containing 1% acetic acid and consecutively applied to cation exchange MCX columns (Waters Cromatografía, S.A.). The fraction containing the acidic ABA, GA_1, GA_4, IAA and tZ hormones was applied through ion exchange WAX columns (Waters Cromatografía, S.A.). The final residue was dissolved in 5% acetonitrile-1% acetic acid, and the hormones were separated using an autosampler and reverse phase UPHL chromatography (2.6 µm Accucore RP-MS column, 50 mm lengthÅ~2.1 mm i.d.; ThermoFisher Scientific Inc., Waltham, MA, USA) with a 5–50% acetonitrile gradient containing 0.05% acetic acid, at 400 µl min 1 during

14 min

The hormones were analyzed with a Q-Exactive mass spectrometer (Orbitrap detector; ThermoFisher Scientific Inc., Waltham, MA, USA) by targeted Selected Ion Monitoring (SIM). The concentrations of hormones in the extracts were determined using embedded calibration curves and the Xcalibur 2.2 SP1 build 48 and TraceFinder programs. The results are the mean of three replicate samples.

2.4. Microscopic analysis

Samples of apical buds were fixed in a karnofsky solution (Glutaraldehyde-paraformaldehyde) and embedded in LR-White resin through a post-fixation in 2% osmium, washing with distilled water, dehydration with increasing ethanol (30, 50, 70, 90), resin infiltration in increasing concentrations (1 part resin + 2 parts ethanol 90, 2 parts resin + 1 part OH 90, 2 parts resin + 1 part OH 100 and finally 100% resin. Polymerization took place in an oven with the temperature set at 60 °C.

Embedded material was sectioned at 2 µm in Ultracut UC6 of Leica with Diamond blade Diatome. The sections obtained were stained with toluidine blue.

Preparations were observed and photographed with a bright field microscope (E600, NIKON). The images were collected using a photographic camera (NIKON digital) attached to the microscope and processed using a Quantiment 570 Image Analysis System (Leica Cambridge, Cambridge, United Kingdom).

2.5. Statistical analysis

Data were subjected to ANOVA analysis and Student's t-test for mean separation using Statgraphics Plus 5.1 software (Statistical Graphics, Englewood Cliffs, NJ). Percentages were arcsin- transformed to homogenize that variance.

3. Results

In the first summer of the experiment, loquat trees induced to flower were exposed to temperatures below 25ºC (average temperature 21.4ºC), i.e. during floral transition (from 25 June to 28 August), suppressed flowering, in contrast to those grown under field conditions (average temperature 27.4ºC) (Table 2). Afterwards, trees transferred to the field did not flower at all.

Similar results were observed in the second year, when trees induced to flower were moved from the field to different indoor controlled thermal conditions. Flowering was suppressed in all trees grown indoors irrespective of the period, except for those grown during the first 15 d of July, which, nonetheless, scarcely flowered and significantly less (13%) than the control trees (Table 3). It is worth noting that just 20 d under controlled temperature variations in early summer, i.e. max/min indoors Tª 7.6ºC/4.1ºC lower, respectively, than in the field during the first two weeks of July, was enough to disrupt the floral bud differentiation process, and 12 more days indoors completely suppressed it (Table 3).

Moreover, a later (27 July-24 August), but slightly shorter, period (28 days) indoors also prevented flowering (Table 3). Conversely, high temperatures (max Tª> 25ºC) during the summer rest period, established between July 15 and 27, promoted the floral transition under Mediterranean climate conditions.

3.1. Effect of temperature on floral meristem identity gene patterns

The *EjTFL1* relative expression pattern in the apical meristem of the trees grown in the field and indoors was opposite. In the former, a sharp decrease up to 3.1-fold was detected in mid-July, whereas in the latter the expression increased gradually up to 2-fold for the same period, remaining almost constant until the end of the studied period in both cases (Fig. 3). Interestingly, the low *EjTFL1* expression detected in the field from mid-July onwards coincided with the increase in temperatures (Fig. 1), and by contrast, constant temperatures below 25ºC indoors allowed the *EjTFL1* expression to increase. The response to indoor conditions was immediate and 8 d after keeping trees at a maximum temperature below 25ºC, the level of *Ej*TFL1 transcript accumulation increased 1.5-fold in contrast to that in the field (Fig. 3). On average, the expression of *EjTFL1* was 6-fold higher for 23ºC- 25ºC thermal variations (indoors) compared to 34ºC – 37ºC (field), and a negative significant correlation ($r = -0.975$; $P < 0.01$) between the maximum daily temperature and *Ej*TFL1 expression was found.

Similarly, when trees were grown at temperatures below 25ºC from 15 July to 27 August, *EjTFL1* relative expression in the meristem dramatically increased, up to 12-fold (Fig. 4A), the response remaining constant up to 15 d later (11 August) at least (Fig. 4A), whereas in the field it remained very low (Fig. 4D).

Table 2
Flowering response of 'Algerie' loquat trees exposed to actual growing conditions (mean field Tª: 27.4ºC) or cold temperature stress (mean indoors Tª: 21.4ºC), during the flower bud differentiation period. A group of 12 trees was transferred from field to indoor conditions (25 June) after floral bud induction and maintained until the end of August (28th), whereas other 12 trees remained in the field. The percentage of vegetative and reproductive shoots was evaluated at flowering (505 BBCH; 20 October). Differences in vegetative and floral shoots are statistically significant ($P < 0.01$).

Indoor period	No. Days indoors	Vegetative shoots (%)	Floral shoots (%)	Average Tª (ºC)		
				Max Tª	Min Tª	Mean Tª
25 June - 28 August	64	100	0	23.7 ± 0,06	19.2 ± 0,04	21.4 ± 0,04
Field	0	3	97	35.4 ± 0,19	20.5 ± 0,18	27.4 ± 0,15

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Table 3
Effect of low thermal regime (average max/min Tª 24.5ºC/18.1ºC) in comparison with a high one (31.3ºC/23.1ºC) during the period of floral transition on the flowering process of 'Algerie' loquat trees. After floral bud induction (25th June), 3 groups of 6 trees each were transferred from the field to the indoor conditions for 20, 32 and 60 days. Another 2 groups of 6 trees each were transferred to indoor conditions the 15th and 27th of July for 40 and 28 days, respectively. The percentage of vegetative and reproductive shoots was evaluated at flowering (505 BBCH; 20th October). Different letters for a given shoot indicate significant differences ($P < 0.01$).

Indoor period	No. Days indoors	Shoots (%)		Temp max/min indoors (ºC)	Temp max/min field (ºC)
		Vegetative	Floral		
25 June - 15 July	20	87 b	13 b	24.2 /18.2	31.8 /22.3
25 June - 27 July	32	100 a	0 a	24.3 /18.3	32.0 /22.8
25 June –24 August	60	100 a	0 a	24.5 /18.1	33.5 /23.0
15 July - 24 August	40	100 a	0 a	24.7 /18.0	33.4 /23.5
27 July - 24 August	28	100 a	0 a	24.8 /18.0	33.2 /23.7
Field	0	2 a	98 c		34.2 / 23.1

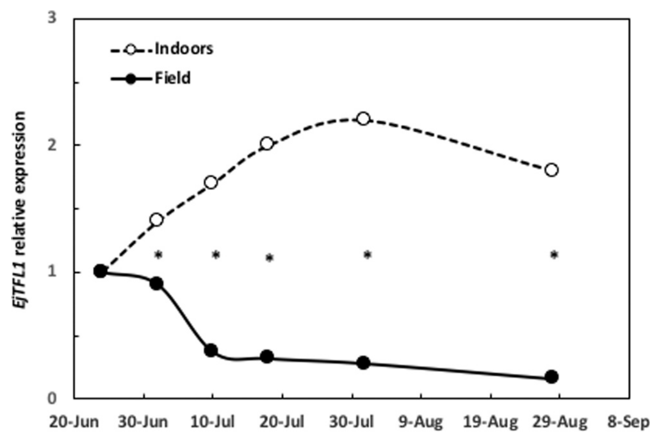

Fig. 3. Expression pattern of the *EjTFL1* gene in apical buds of 'Algerie' loquat trees grown during summer under field conditions (30–36ºC day/21–26ºC night), and indoor conditions (24–25ºC day/18–19ºC night) between 25June and 28 August. Data are the mean of 3 biological replicates and 3 technical replicates each. In all cases, bars of SE are smaller than the symbol size. * for a given data indicates significant differences ($P < 0.05$).

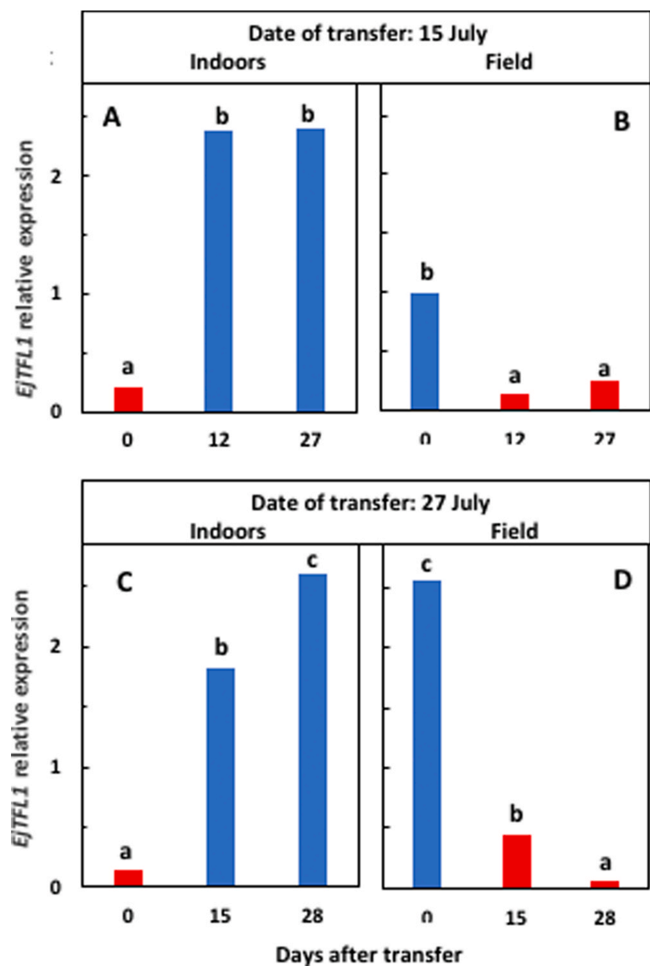

Fig. 4. The effect of temperature-controlled conditions (indoors: 24–25ºC day/ 18–19ºC night and field: 30–36ºC day/21–26ºC night) during two times intervals (15 July-24 August: A, B) and (27 July-24 August: C, D) on *EjTFL1* gene expression in apical buds of 'Algerie' loquat trees. Data are the mean of 3 biological replicates and 3 technical replicates each. In all cases, SE cannot be plotted because they are too small. Different letters for a given treatment indicate significant differences ($P < 0.05$).

But when trees growing indoors (Tmax: 24.2ºC) were returned to the field (Tmax: 31.8ºC) on 15 July, the *EjTFL1* relative expression sharply decreased, up to 7-fold (Fig. 4B). The response was also immediate and 12 d after ≈ 8ºC temperature increase, the *EjTFL1* expression decreased by 80%, approx., remaining low over time (Fig. 4B), and causing the trees to bloom, although just barely (Table 3).

Similar results were observed when the trees were transferred from the field to indoors on 27 July (Fig. 4C), or from indoors to the field (Fig. 4D); *EjTFL1* expression increased 15.7-fold and decreased 9.6-fold on average, respectively, but in this case trees did not bloom. Therefore, heating trees reduced *EjTFL1* transcript levels, regardless of the date the trees were moved to the field (Fig. 4B and D), but depending on the time the tree remains at T[a] lower than 25ºC, it may not be enough to allow the tree to bloom.

In the first year, the time-course of *EjAP1* and *EjLFY* accumulation in the terminal meristem of the trees grown under the field conditions revealed a sharp increase peaking in late July (30 July) and mid August (19 August), respectively (Fig. 5), the latter coinciding with the first visual microscopic signals of floral bud differentiation (Fig. 6), followed by a sharp decrease at mid and end-August, respectively. On the contrary, there was no expression of the transcript of either *EjAP1* or *EjLFY* meristem identity gene in trees grown indoors which remained constant throughout the sampling period (Fig. 5).

Histologically, apical buds showed no signs of floral differentiation in the meristem of the trees grown under field conditions up to the beginning of August (the 8th). In our experiment, the first signs of morphological floral structures were observed on 14th August coinciding with the peak of *EjLFY* expression (Fig. 5), becoming trimerous forms, and the inflorescence axis structures were visible on the August 20th. Those from apical buds of the trees grown under indoor conditions remained in a vegetative stage up to this date (Fig. 6). These results are in accordance with the floral meristem identity gene pattern presented

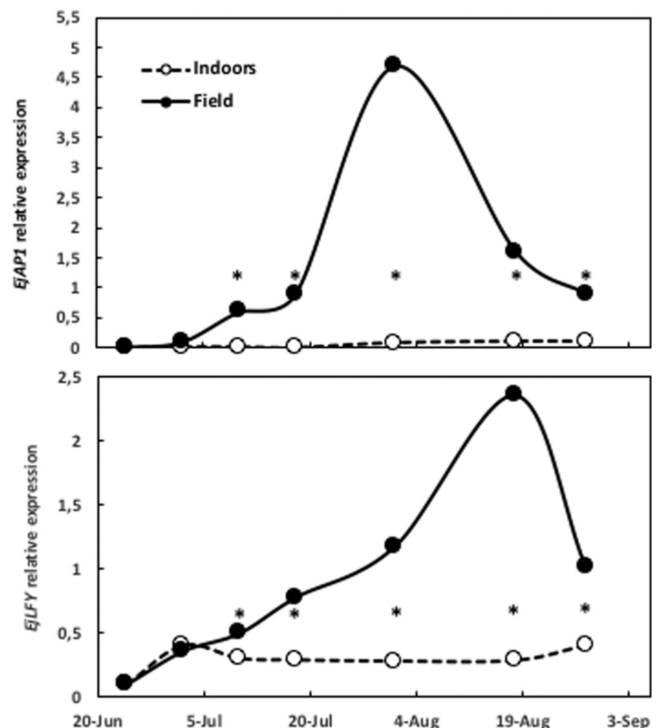

Fig. 5. Expression pattern in summer of the *EjAP1* and *EjLFY* genes in apical buds of 'Algerie' loquat trees grown in the field (30–36ºC day/21–26ºC night) and indoors (24–25ºC day/18–19ºC night). Data are the mean of 3 biological replicates and 3 technical replicates each. In all cases, bars of SE are smaller than the symbol size. * for a given data indicates significant difference ($P < 0.05$).

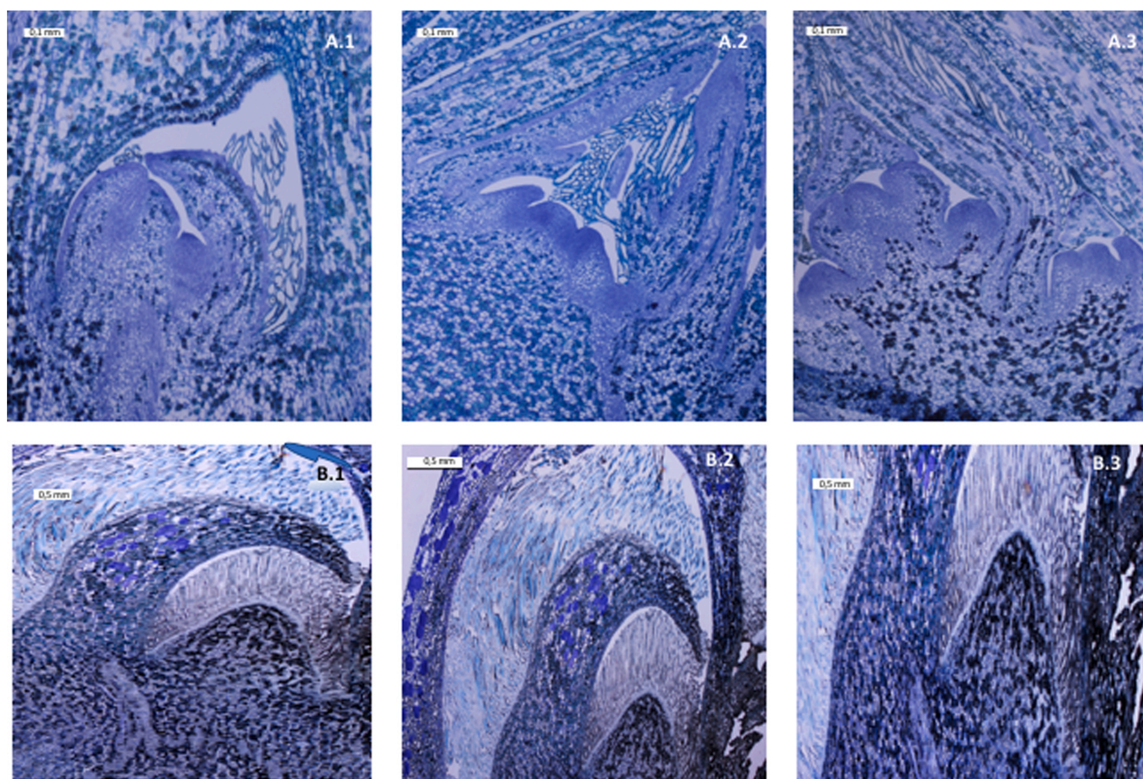

Fig. 6. Histological observation of the apical buds of 'Algerie' loquat during the inflorescence initiation. A.1, A.2, A.3: microscopic longitudinal sections of apical buds of trees grown under the field conditions (30–36ºC day/21–26ºC night). B.1, B.2, B.3: microscopic longitudinal sections of apical buds of trees grown indoors (24–25ºC day/18–19ºC night). One apical bud per tree of 6 trees was sampled in both cases. Buds were collected on 8th (A.1, B.1), 14th (A.2, B.2) and 20th (A.3, B.3) August. The most representative images for each date and group of trees are shown.

above (Fig. 5), suggesting mid – August as the most feasible time for floral initiation in loquat under Mediterranean climate conditions.

Similarly to year 1, when the trees were transferred indoors on July 15th, transcripts of *EjAP1* in terminal buds reduced expression 2-fold, approximately, after 12 or 27 d (max T: 24.2ºC) (Fig. 7A), and also when transferred 12 d later (27 July), although to a lower extent (Fig. 7C). In both cases, reducing the temperature by approximately 8ºC during mid to end July caused a 50% decrease in *EjAP1* expression and trees did not flower at all (Table 3).

On the contrary, when trees were moved from indoor (max T: 24.2ºC) to field conditions (max T: 32.1ºC on average), the *EjAP1* expression increased regardless of the date (Figs. 7B and 7D), the response increasing over time up to 3.5 and 2.3- fold, twenty-seven and twenty-eight days after being transferred to the field on the 15th or 27th of July, respectively (Figs. 7B and 7D). In spite of the increase in the *EjAP1* (Figs. 7B and 7D) and the decrease in the *EjTFL1* (Figs. 4B and 4D) expression in the field, the former barely flowered whereas the latter, which remained 12 more days indoors, did not flower at all (see Table 3).

Similar results were observed for *EjLFY*. The increase and decrease in temperature paralleled the increase and decrease in gene expression (Fig. 8) in a similar proportion to that of *EjAP1*.

3.2. *Effect of temperature on plant hormones content*

Thermal conditions modified significantly the ABA content. The time-course of ABA concentration in the meristem of trees grown in the field and indoor was opposite. In the former, a sharp decrease in ABA concentration (78.5%) was observed 35 d after transferred indoors (the end of July), remained almost constant until one month later (1200 ng g^{-1} DW, on average), whereas in the latter it gradually increased until reaching the maximum (8941 ng g^{-1} DW) at the end of August, becoming 7.5-fold higher than in field conditions (Fig. 9). The response to the temperature change was not immediate, while ABA concentration was almost constant (≈ 4000 ng g^{-1} DW) during the first 15 d after the trees were transferred indoors. But one week later (18 July), ABA concentration in the meristem of trees grown in the field was 1.6-fold lower than that of those grown indoors (Fig. 9).

No differences in the time-course of gibberellin (GA_1, GA_4), cytokinin (tZ) and auxin (IAA) were found between meristems of the trees grown indoors and in the field (data not shown).

Similarly to the first year, when trees were grown at temperatures lower than 25ºC from 15 July onwards, ABA concentration in the meristem gradually increased up to 2.8-fold after 27 d indoors (Fig. 10A). But when the trees were moved from indoors to the field on 15 July, the ABA concentration dropped rapidly to 2.9-fold after 12 d (Fig. 10B), remaining almost constant during the next 15 d (Fig. 10B).

Similarly, when the trees were moved indoors from the field (Fig. 10C), or from indoors to the field at the end of July (Fig. 10D), the ABA concentration increased 5.5-fold and decreased 3.3-fold, on average, respectively.

It is important to note the ABA concentration in the meristems of the trees grown under the field conditions decreased from 489.1 ng g^{-1} DW on 15th of July (Figs. 10A) to 289.1 ng g^{-1} DW on 27th of July (Fig. 10C), as the temperature rose 0.7 ºC.

As for year one, no differences in the time-course of gibberellin (GA_1, GA_4), cytokinin (tZ) and auxin (IAA) were found between meristems of the trees grown indoors and those grown in the field (data not shown).

4. Discussion

In perennial fruit tree species, the transition to the reproductive phase is regulated by a network of signalling pathways of flowering-time genes responding to environmental signals as well as internal cues (Levy

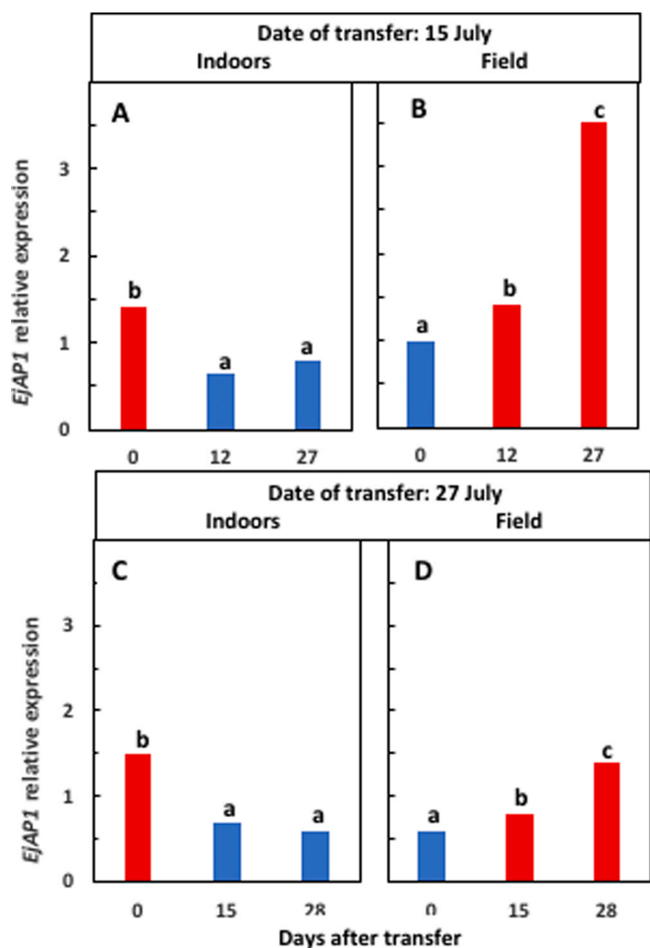

Fig. 7. The effect of temperature-controlled conditions (indoors: 24–25ºC day/18–19ºC night, and field: 30–36ºC day/21–26ºC night) and date of transfer (15 July-24 August: A, B) and (27th July-24th August: C, D) on *EjAP1* gene expression in apical buds of 'Algerie' loquat trees. Data are the mean of 3 biological replicates and 3 technical replicates each. In all cases, SE cannot be plotted because they are very small. Different letters for a given treatment indicate significant differences ($P < 0.05$).

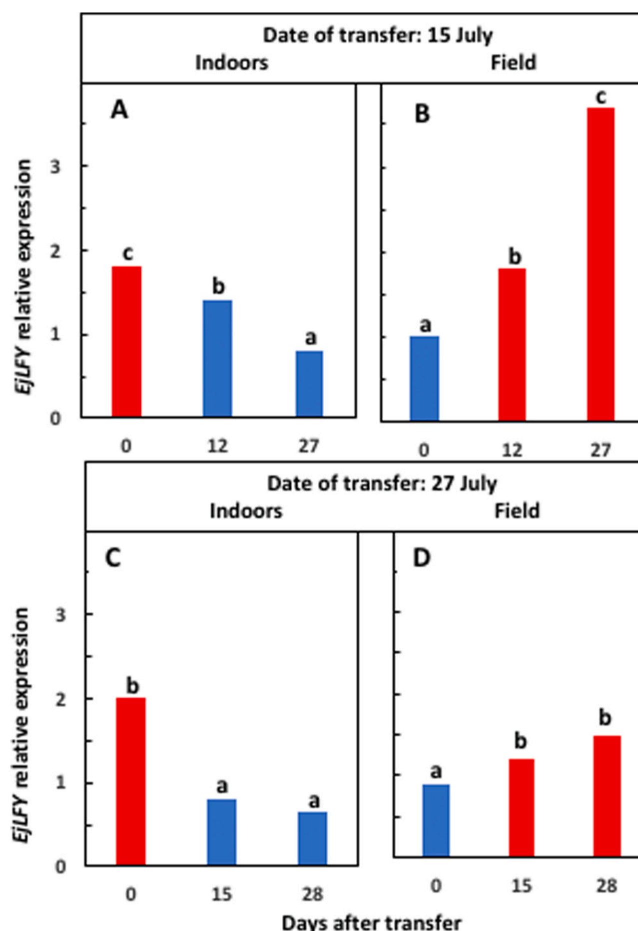

Fig. 8. The effect of temperature-controlled conditions (indoors: 24–25ºC day/18–19ºC night, and field: 30–36ºC day/21–26ºC night) during different time intervals (15 July-24 August: A, B) and (27 July-24 August: C, D) on *EjLFY* gene expression in apical buds of 'Algerie' loquat trees. Data are the mean of 3 biological replicates and 3 technical replicates each. In all cases, SE cannot be plotted because they are very small. Different letters for a given treatment indicate significant differences ($P < 0.05$).

and Dean, 1998). These pathways eventually converge by regulating a large group of flowering time genes like *FT, SOC1*, *AP1 and LFY* (Pin and Nilsson, 2012). Activation of these genes triggers the transition to flowering (Kaufmann et al., 2010), whereas that of *TFL1* is involved in maintaining the meristem indeterminate, repressing flowering by preventing the expression of *AP1* and *LFY* (Boss et al., 2004).

Notwithstanding, there are notable differences in flowering patterns between these species. This is the case of deciduous and evergreen Rosaceae species, for which the time-course of floral bud induction-differentiation differs, indicating that the factor triggering annual flowering may also differ. Thus, in the former the floral initiation begins in the preceding summer broken by winter dormancy after which the bud sprouts and flowers. In the latter, particularly in loquat, the meristem grows during summer until it is differentiated into a panicle in early autumn, so the flowering is a continuous process that is not interrupted by winter dormancy. In both cases, photoperiodic signals do not seem to play a large (if any) role inducing flowering (Koffler et al., (2019)), but the ambient temperature does, and a period of dormancy imposed by high temperatures seems necessary for floral bud differentiation. Heide et al. (2020) showed a range of 18°C-21°C as the optimum temperature for floral bud initiation in apple, whereas at 12 °C flowering is repressed by constraining the growth, and at 27 °C flowering is blocked by inhibiting the floral initiation itself. Sonsteby and Heide

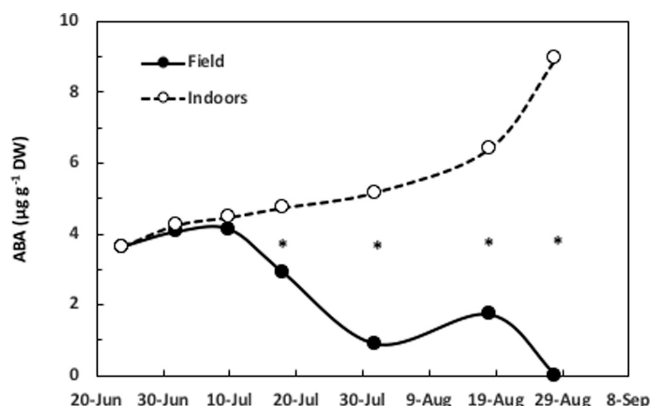

Fig. 9. Time-course of ABA concentration during summer in apical buds of 'Algerie' loquat trees grown in the field (30–36ºC day/21–26ºC night) and indoor conditions (24–25ºC day/18–19ºC night). Trees were transferred indoors on 25 June. Data are the mean of 3 biological replicates and 3 technical replicates each. In all cases, bars of SE are smaller than the symbol size. Normalization was performed to the first sample date. * indicates significant differences for a given data ($P < 0.05$).

(2019) demonstrated that growth cessation and floral initiation

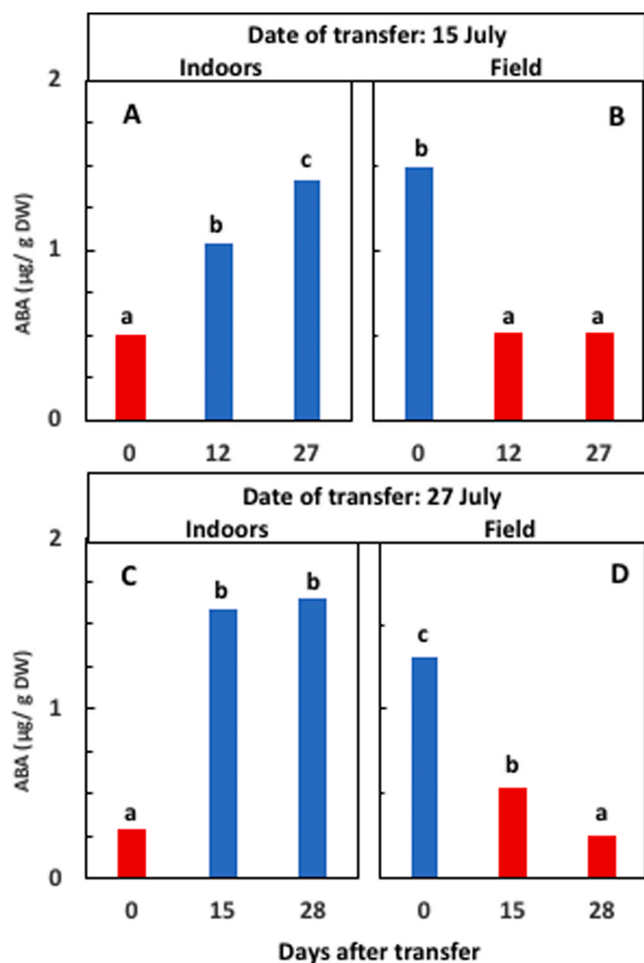

Fig. 10. The effect of temperature-controlled conditions (indoors: 24–25ºC day/18–19ºC night, and field: 30–36ºC day/21–26ºC night) during different time intervals (15 July-24 August: A, B) and (27 July-24 August: C, D) on ABA content in apical buds of 'Algerie' loquat trees. Data are the mean of 3 biological replicates and 3 technical replicates each. SE cannot be plotted because they are very small. Different letters for a given treatment indicate significant differences ($P < 0.05$).

occurred with increased summer temperatures in sweet cherry; low temperatures (12–15 °C) advanced floral initiation compared with higher temperatures (21 °C), and high temperatures delayed floral initiation but not differentiation.

This suggests that the floral pattern is partially driven by temperature, preventing the expression of floral promoting genes (Kumar and Wigge, 2010) and/or increasing floral repression as a result of low temperatures (Lee and Lee, 2010; Posé et al., 2013). In Rosaceae, functional characterization of *TFL1* is confirmed as the main floral repressor (Koskela et al., 2012), preventing the ectopic expression of the meristem identity genes *AP1* and *LFY* in the shoot apex and, therefore, preventing the meristem from the floral identity (Bradley et al., 1997). Moreover, since initial *TFL1* mRNA levels may be needed to inhibit *LFY* effects and, therefore, to be a marker for a switch to bolting inflorescences stages (Ratcliffe et al., 1999), this could be the reason why our experiment detected a high *TFL1* expression during late June – early July in the SAM, which correlates with vegetative to reproductive transition, as occurs during the annual cycle in other Rosaceae (Koskela et al., 2012). And on the contrary, the minimum *EjTFL1* expression in the SAM was detected from mid-July onwards, coinciding with the increase in temperature and with the maximum *EjAP1* and *EjLFY* expression, and also with the first microscopic symptoms of floral bud differentiation (see Fig. 4). In fact, the silencing of *TFL1* homologues in apple and pear causes perpetual flowering (Flachowsky et al., 2012; Freiman et al., 2012), whereas in our experiments a significant increase in *EjTFL1* expression by reducing indoor temperatures (21.3 °C) with respect to the field (28.5 °C) caused perpetual vegetative growth, and the trees did not flower at all, in agreement with Kim et al. (2013).

In fact, long-term exposure of loquat trees to temperatures close to 21ºC during flowering transition (from 15 July) up-regulates the floral repressor which is crucial in the inhability of the meristem to flower. In addition, our results showed that the expressions of *EjAP1* and *EjLFY* were up-regulated after the down-regulation of *EjTFL1*, indicating that they probably function downstream to the *EjTFL1*, as in model plants (Hanano and Goto., 2011). In this sense, our results suggest that the *EjTFL1* gene plays a major role in plant's temperature-response in addition to its function in vegetative growth found in apple (Hättasch et al., 2008; Kotoda et al., 2006; Mimida et al., 2009). Accordingly, when trees were grown under low temperature conditions (average Tª<25 °C), from 15 July onwards, *EjTFL1* relative expression increased up to 12-fold, regardless of the time remained indoors (Fig. 4A). Results suggest that higher temperatures in July are responsible for the lack of the *EjTFL1* relative expression, thus allowing for the *LFY* and *AP1* expression and floral bud differentiation.

In spite of this, *EjTFL1* expression might also be regulated by an endogenous signal rather than the temperature. Bearing this in mind, cool temperatures during July would be the matter of choice for future gene silencing strategies, as proposed in apple (Weigl et al., 2015). Previous reports suggested that *LFY* is known to be the target of several endogenous signals, such as gibberellin (Blázquez and Weigel, 2000; Mutasa-Göttgens and Hedden, 2009), and fruit up-regulates *CsTFL* relative expression (Muñoz-Fambuena et al., 2012) by generating an auxin signal in the bud and apical meristem, repressing the activity of *CsAP1* and *CsFLY* causing lack of flowers in *Citrus* (Haim et al., 2021).

In both, annual and perennial plants, the application of benzyladenine replaces the environmental inducing signal (Srinivasan and Mullins, 1979; Bernier (1988); Tisserat et al., 1990), an optimal concentration, depending on the species, being required to stimulate meristematic ability in the bud flower. In addition, sugar and CK, ABA and GA related genes have been shown to be involved in floral induction in apple (Xing et al., 2015), and biosynthesis and signaling genes related to auxin, ABA, and ethylene were also identified in pear as *PpTFL1* co-expressive genes (Bai et al., 2017). Our results show that ABA, IAA, tZ and gibberellin (GA_1, GA_4) content decrease during the floral transition (mid July - mid August) paralleling *EjTFL1* expression (Figs. 3 and 9). This suggests that the expression of the plant hormone-related genes concomitantly changes in a synergistic way to *EjTFL1*, as for pear (Bai et al., 2017). Conversely, the ABA content increased when trees were grown indoors, i.e. under 25 °C constant temperature, and *EjTFL1* expression grew in parallel during the same period (Figs. 3 and 9), whereas that of IAA and gibberellin (GA1, GA4) decreased. In *Arabidopsis*, ABA delays flowering of plants grown in salt in a DELLA-dependent pathway, but *FT* and *SOC1* transcript levels were not affected (Achard et al., 2006). Similarly, in our experiments ABA content was high in the apex of shoot that did not flower in July, i.e. growing indoors at low temperatures, suggesting that, in this case, the inhibition of flowering by ABA acts through ambient temperature, converging at the floral integrators. But the fact that the increase in *EjTFL1* expression predates the increase in ABA content must be questioned. Thus, the *TFL1-FD* complex directly represses the ABA biosynthetic genes (Zhu et al., 2020), and a competition between *FT* and *TFL1* modulating ABA levels or sensitivity in the shoot meristem cells has been suggested (Martignago et al., 2020). Furthermore, our results show slight delay in decreasing ABA content with regard to the *TFL1* expression when the trees were transferred indoors. By contrast, in Satsuma mandarin ABA applied locally to the bud in the floral bud inductive period inhibited flowering on trees that bloom profusely (Garcia-Luis et al., (1986)), and in *Arabidopsis* FLC transcript levels increase in response to ABA applications (Wang et al., 2013). Consistent with this ABA-FLC regulation,

ABA-hypersensitive mutants display increased accumulation of FLC and are late-flowering compared to the wild type.

In apparent contrast, knowing the ABA is considered as a stress-related hormone, the notable increase observed in the trees grown indoors could be associated with an adaptive strategy for new low temperature conditions. Moreover, ABA not only activates the genes associated with low temperatures, but also a wide array of genes linked to drought, salinity (Busk and Pagès (1998)) and the closure of stomates (Shohat et al., 2020). Accordingly, ABA in the floral process remains unclear and further studies are needed to understand which genetic adjustment coordinates ABA sensitivity and flowering time.

5. Conclusion

In summary, in loquat when flower intiation occurs in early mid – summer (from late-July to late-August), low levels of *EjTFL1* transcripts were detected in the terminal shoot apex. This correlated with the high temperature and coincided with the cessation of the shoot apex meristem to produce new leaf primordia, and shoot growth stops. Afterwards, when temperatures drop and the terminal shoot apex reactivates, floral differentiation begins. Indeed, under lower temperatures (25 °C), like those our indoor conditions, during the same period, shoot apex did not stop growing and a higher *EjTFL1* transcription level was registered, preventing the bud to flower. Our results also correlate negatively ABA content with flowering, and suggest that the ABA concentration in the buds might be a regulator signal for the floral bud differentiation in loquat. Enhancing its biosynthesis by reducing growing temperatures impedes floral bud differentiation positively correlating with *EjTFL1* expression.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CrediT authorship contribution statement

C.R. and M.A. conceived and designed the research; A.G.-L., C.M., C. R., and M.A. performed the experiments and conducted the work; A.M.-F. carried out the biochemical analyses; C.M. and M.A. analysed the data and wrote the manuscript with contributions from all authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

We thank IBMCP-UPV (Valencia, Spain) for providing the greenhouse, Dr. E. Carrera, from the IBMCP-UPV, for plant hormones analyses, and Dr. D. Westall for editing the manuscript.

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