Dottorato in 'Frutticoltura Mediterranea'
Dipartimento di Scienze Agrarie e Forestali
AGR/03 - Arboricoltura generale e Coltivazioni arboree

Departamento de Producción vegetal

# Temperature regulating floral bud differentiation in loquat (*Eriobotrya japonica* Lindl.). Hormonal and genetic aspects

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February 2017

CICLO XXIX

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*‘Lo que sabemos es una gota de agua; lo que ignoramos es el océano’*

Isaac Newton

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# *Abbreviations*

**ABA.** Abscisic acid

**AGL24**. AGAMOUS-LIKE 24

**AP1**. APETALA 1

**bZIP.** Basic Leucine Zipper

**CAL**. CAULIFLOWER

**ChIP**. Chromatin immunoprecipitation

**CK**. Cytokinin

**CO**. CONSTANS

**CR**. Chilling requirement

**DE**. Drought escape

**DHZ**. Dihydrozeatin

**DNA**. Deoxyribonucleic acid

**DW**. Dry weight

**EF**. Efficiency

**EjAP1**. Eriobotrya japonica APETALA 1

**EjFT**. Eriobotrya japonica FLOWERING LOCUS T

**EjLFY**. Eriobotrya japonica LEAFY

**EjTFL1**. Eriobotrya japonica TERMINAL FLOWERING 1

**FD**. FLOWERING LOCUS D

**FLC**. FLOWERING LOCUS C

**FLD**. FLOWERING LOCUS D

**FMI**. Floral meristem identity

**FT**. FLOWERING LOCUS T

**FUL**. FRUITFULL

**GA**. Gibberellin

**GI**. GIGANTEA

**HPLC.** High-performance liquid chromatography

**HR**. Heat requirement

**IAA.** Indole acetic acid

**iP**. Isopentenyl adenine

**LC/MS/MS.** Liquid chromatography–mass spectrometry

**LD**. Long-days

**LD**. LUMINIDEPENDENS

**LFY**. LEAFY

**LSD**. Least significant difference

**MADS.** MCM1/AGAMOUS/DEFICIENS/SRF

**PEBP**. Phosphate ethanolamine binding protein

**PIF**. PHYTOCHROME INTERACTING FACTOR

**RKIP**. Raf kinase inhibitor protein

**RMSE**. Root means squared error

**RNA**. Ribonucleic acid

**RT-PCR/qRT-PCR.** Real time – polymerase chain reaction

**SAM**. Shoot apical meristem

**SD**. Short-day

**SE.** Standard error

**SIM.** Selected Ion Monitoring

**SOC1**. SUPRESSOR OF OVEREXPRESSION OF CO1

**SVP.** SHORT VEGETATIVE PHASE

**TFL1**. TERMINAL FLOWERING 1

**TIBA**. Triiodobenzoic acid

**TSF.** TWIN SISTER OF FT

**tZ.** Trans-zeatina

**UPLC-MS/MS.** Ultra-high pressure liquid chromatography, mass spectrometry

**VIN3**. VERNALIZATION INSENSITIVE3

**VRN**. VERNALISATION

# *Abstract*

## Abstract

In loquat, apex of a current shoot changes from vegetative to reproductive stage during summer, i.e. under high temperature conditions. Indeed, just before floral bud differentiation, a decline in the growth rate due to high temperature takes place. The aim of this work is to study the role of this 'summer rest period' on the apex transition from vegetative to reproductive stage. For this purpose 1) sprouting of secondary shoots was promoted at different times, removing the main shoot, before, during and after floral bud differentiation occurred and 2) groups of trees were shifted to a greenhouse under average maximum temperature not exceeding 25 ° C during different periods from June to October.
Floral bud differentiation was evaluated. *LEAFY (LFY), APETALA (AP1), TERMINAL FLOWERING 1 (TFL1)* and *FLOWERING LOCUS T (FT1)* expression and hormonal content in abscisic acid (ABA), gibberellins (GAs), indoleacetic acid (IAA) and cytokinins (CKs) were analyzed in bud collected during the summer.

Results suggest that the date of shoot apex removal determining floral bud differentiation of new shoots, so that the percentage of the new reproductive shoots reduced with the delaying of apex removal. On the other hand, maximum average temperature not exceeding 25 ° C prevented floral bud differentiation. Buds of the trees under indoors conditons displayed lower expression of identity floral genes *EjLFY* and *EjAP1* than buds of trees grown in field. On the contrary, the floral repressor *EjTFL1* and *EjFT1* gene expressed higher in buds of the trees grown indoors. Time-course of ABA decreased in buds of trees grown in field during studied period while in buds of trees under greenhouse conditions displayed a growing trend. Time-course of GAs, IAA and CKs concentrations did not show remarkable differences between buds of trees growing under field and indoors conditions. Accordingly, 1) secondary shoots emerged from mid- August are unfitness to flower and 2) maximum average tempertature 25±1 °C during the summer prevents floral bud differentiation, enhances ABA biosynthesis, reduces *EjLFY* and *EjAP1* expression and enhance *EjTFL1* expression in the apex.

**Riassunto**

Il nespolo del Giappone differenzia le sue gemme durante l'estate, dopo un periodo di rallentamento della crescita vegetativa legato alle alte temperature, noto come “periodo di riposo estivo”. Lo scopo di questa tesi è stato quello di studiare l'influenza di detto riposo estivo nella differenziazione fiorale di questa specie. A tal scopo si è disegnato un esperimento che ha previsto l’eliminazione degli apici in distinte date, tra luglio e settembre, prima, durante e dopo il periodo di differenziazione, per indurre la produzione di germogli secondari. Allo stesso tempo, si è realizzato un altro esperimento in cui gli alberi sono stati divisi in diversi gruppi e trasferiti in serra, ad una temperatura media-massima di 25 °C, durante periodi di diversa durata. Si è dunque proceduto a valutare la differenziazione fiorale ed inoltre ad analizzare l’espressione di alcuni geni legati alla fioritura: *LEAFY (LFY), APETALA (AP1), TERMINAL FLOWERING 1 (TFL1)* e *FLOWERING LOCUS T (FT1)* ed il contenuto ormonale di acido abscissico (ABA), acido gibberellico (GA), acido indolacetico (AIA) e citochinine (CK) degli apici campionati durante il periodo estivo.

I risultati ottenuti indicano che la data di rimozione degli apici condiziona la differenziazione fiorale dei germogli anticipati in modo tale che, la data di rimozione è inversamente proporzionale alla percentuale di germogli fiorali. Inoltre, condizioni di temperatura media-massima non superiore a 25 °C impediscono totalmente la differenziazione fiorale. Negl’apici degl’alberi che sono stati mantenuti in queste condizioni il livello di espressione dei geni di identità florale, *EjLFY* ed *EjAP1*, è risultato essere molto inferiore rispetto a quello degl’alberi in condizioni di campo. Al contrario, l'espressione del repressore della fioritura *EjTFL1* e del gene *EjFT1* è risultata maggiore negl’apici degl’alberi in serra. D'altra parte, durante il periodo di studio, il contenuto di ABA endogeno è diminuito negl’alberi in campo mentre negli alberi in serra ha avuto una tendenza crescente. Tuttavia, non sono state rilevate differenze tra le concentrazioni di GAs, AIA e CKs negli apici degli alberi in campo e in serra. Di conseguenza, 1) gli apici germogliati dopo metà agosto non sono stati capaci di fiorire e 2) l'assenza di alte temperature estive promuove l'accumulo di ABA, aumenta l'espressione del gene repressore (*EjTFL1*) e

riduce l'espressione dei geni d’identità fiorale (*EjLFY* e *EjAP1*) negl’apici di nespolo, impedendone la differenziazione fiorale.

**Resumen**

El níspero japonés diferencia sus yemas durante el verano, después de un periodo de ralentización del crecimiento vegetativo ligado a las altas temperaturas que se conoce como reposo estival. El objetivo de esta tesis fue estudiar la influencia de la parada estival en la diferenciación floral de esta especie. Para ello se diseñó un experimento en el que se forzó la brotación de brotes anticipados eliminado el ápice principal en diferentes fechas entre julio y septiembre, antes, durante y después de la parada estival. Paralelamente se diseñó otro experimento en el que se cambiaron las condiciones climáticas a grupos de árboles manteniéndolos en un invernadero a una temperatura máxima media de 25 °C durante diferentes periodos de diversa duración. Se evaluó la diferenciación floral y se analizó la expresión de los genes relacionados con la floración *LEAFY (LFY), APETALA (AP1), TERMINAL FLOWERING 1 (TFL1)* and *FLOWERING LOCUS T (FT1)* y el contenido hormonal en ácido abscisico (ABA), giberelinas (GAs), ácido indolácetico (AIA) y citoquininas (CKs) en yemas terminales muestreadas a lo largo del verano.

Los resultados indican que la fecha de brotación modifica la diferenciación floral de los brotes anticipados siendo el porcentaje de brotes reproductivos inversamente proporcional a la fecha de eliminación del meristemo. Del mismo modo unas condiciones de temperatura máxima no superior a 25 °C impidieron la diferenciación floral. Las yemas de los árboles que estuvieron bajo dichas condiciones mantuvieron unos niveles de expresión de los genes de identidad floral, *EjLFY* y *EjAP1*, mucho menor que la de los árboles en condiciones de campo. Por el contrario, la expresión del represor *EjTFL1* y del gen *EjFT1* fue mayor en los árboles en invernadero. Por otro lado, el contenido endógeno de ABA descendió en los árboles situados en el campo durante el periodo de estudio mientras que en los árboles situados en el invernadero tuvo una evolución ascendente. Las concentraciones de GAs, AIA y CKs no mostraron prácticamente diferencias entre los ápices de los árboles mantenidos en campo y en invernadero. De acuerdo con ello, 1) los brotes anticipados surgidos a partir de mitad de agosto son incapaces de florecer y 2) la ausencia de altas temperaturas del verano promueve la acumulación de ABA, aumenta la expresión del gen represor (*EjTFL1*) y reduce la expresión de los genes de

identidad floral (*EjLFY* y *EjAP1*) en yemas de níspero impidiendo su diferenciación floral.

## Resum

El nispro japonés diferència les seus gemmes durant l'estiu, després d'un període d'alentiment del creixement vegetatiu lligat a les altes temperatures que es coneix com repòs estival. L'objectiu d'aquesta Tesi va ser estudiar la influència de la parada estival en la diferenciació floral d'aquesta espècie. Per a això es va dissenyar un experiment en què es va forçar la aparició dels brots anticipats eliminat l'àpex principal en diferents dates entre juliol i setembre, abans, durant i després de l'aturada estival. Paral·lelament es va dissenyar un altre experiment en què es van canviar les condicions climàtiques a grups d'arbres mantenint-los en un hivernacle a una temperatura màxima mitjana de 25 °C durant diferents períodes de diversa durada. Es va avaluar la diferenciació floral i es va analitzar l'expressió dels gens relacionats amb la floració *LEAFY (LFY), APETALA (AP1), TERMINAL FLOWERING 1 (TFL1)* and *FLOWERING LOCUS T (FT1)* i el contingut hormonal en àcid abscísic (ABA) , gibberel·lines (GAs), àcid indolacètic (AIA) i citoquinines (CKs) en gemmes terminals mostrejades al llarg de l'estiu.
Els resultats indiquen que la data de brotació modifica la diferenciació floral dels brots anticipats i el percentatge de brots reproductius es inversament proporcional a la data d'eliminació del meristema. De la mateixa manera unes condicions de temperatura màxima no superior a 25 ° C varen impedir la diferenciació floral. Les gemmes dels arbres que van estar sota aquestes condicions van mantenir uns nivells d'expressió dels gens d'identitat floral, *EjLFY* i *EjAP1*, molt menor que la dels arbres en condicions de camp. Per contra, l'expressió del repressor *EjTFL1* i del gen *EjFT1* va ser més gran en els arbres en hivernacle. D'altra banda, el contingut endogen d'ABA va baixar en els arbres situats al camp durant el període d'estudi mentre que en els arbres situats a l'hivernacle va tenir una evolució ascendent. Les concentracions de GAs, AIA i CKS no van mostrar pràcticament diferències entre els àpexs dels arbres mantinguts en camp i en hivernacle. D'acord amb això, 1) els brots anticipats sorgits a partir de meitat d'agost són incapaços de florir i 2) l'absència d'altes temperatures de l'estiu promou l'acumulació d'ABA, augmenta l'expressió del gen repressor (*EjTFL1*) i redueix l'expressió dels gens d'identitat floral

(*EjLFY* i *EjAP1*) en gemmes de nispro del Japó impedint la seva diferenciació floral.

# *Introduction*

# 1. Flowering

Flowering in higher plants involves the transition of a vegetative meristem into a floral meristem including the necessary development for the irreversible commitment by the meristem to produce a flower or an inflorescence (Kinet, 1993).

The moment of flowering is a key step in the life cycle of plants to maximize reproductive success in a range of environments. In a broad sense, the transition from vegetative to inflorescence shoot meristem is controlled by environmental signals, including photoperiod and temperature, that reflect seasonal changes by an intrinsic mechanism for generating age-related changes in the development and hormone content of the plant shoot like juvenile phase, and by, finally, a system of flowering time genes. The duration of this juvenile phase varies dramatically between species. In trees, it may last many years while annuals become competent to flower after forming just a few leaves.

These changes are related to the acquisition of the ability to flower and the genetic approaches in Arabidopsis have provided access to genes whose products control flowering in response to seasonal changes, contributing greatly to understanding the molecular mechanism related to floral development.

## 1.1. The flower bud induction, flower bud initiation and flower bud differentiation

The flowering process during the adult stage of a higher plant consists of several discrete phases: flower bud *induction*, flower bud *initiation*, flower bud *differentiation* and the *anthesis*.

*Flower bud induction* process is associated with environmental factors and results in the commitment of meristematic cells to form reproductive structures. During this period, the induction stimulus is perceived at the leaves and the genes required for flower development are turned on. As well as, nutrient, hormone and protein metabolism changes inside the bud.

*Flower bud initiation* involves the anatomical and histological transition of vegetative meristems to floral meristems (Davenport, 1990), but no visible morphological differences are observed.

*Flower bud differentiation* is characterized by the morphological differences and the development of the primordia of floral organs. The time of initiation of differentiation varies by the species and environmental conditions. Following differentiation, the rate of flower bud development also depends on climatic conditions (Guardiola, 1997).

*Florigen* is a hypothetical leaf-produced signal that induces floral initiation at the shoot apex. Recent progress in Arabidopsis has led to the suggestion that *FLOWERING LOCUS T* (*FT*) or its product is the mobile flower-inducing signal that moves from an induced leaf through the phloem to the shoot apex, being *FT* the main, if not the only, component of the universal *florigen* (Abe *et al.,* 2005; Huang *et al.,* 2005; Wigge *et al.,* 2005). Moreover, the functions of *FT* and its orthologues are highly conserved among unrelated species. In plants, seasonal changes as temperature and day length are perceived in leaves, which initiate long-distance signalling that induces flowering at the shoot apex. In Arabidopsis, activation of *FT* transcription in leaf vascular tissue induces flowering without activating an intermediate messenger in leaves. In addition, under inductive conditions, *FT* expression in the leaves is not stably maintained but the activation of *FT* in a single leaf is sufficient to induce flowering (Corbesier *et al.,* 2007).

A basic assumption of the florigen is a universal signal for flowering is supported by interspecific grafting experiments. But grafting has been limited by incompatibility between unrelated species and by the fact that monocots are not amenable to grafting. However, these barriers can now be overcome by 'transplanting' the *FT* gene (and its orthologues) into unrelated species. Examples of species in which premature flowering were induced by ectopic expression of *FT* or an orthologue are tobacco (Lifschitz *et al.,* 2006) or wheat (Yan *et al.,* 2006). However, there are examples of grafting experiments in which the receptor failed to flower (Zeevart, 1976). Anyway, the activation of the transcription of *FT-like* genes in leaves has been observed in other species, and appears to be a highly conserved aspect of floral induction. Expression of such genes has been shown in rice (Komiya *et al.,* 2008), barley (Faure *et al.,* 2007), poplar (Böhlenius *et al.,* 2006; Hsu *et al.,* 2011), tomato (Lifschitz *et al.,* 2006),

apple (Kotoda *et al.,* 2010) and *Citrus* (Nishikawa *et al.,* 2007; Muñoz-Fambuena *et al.,* 2011). In loquat, two *FT* orthologues, *EjFT1* and *EjFT2,* have been isolated. Expression analysis in leaves suggest that the gene *EjFT1* is probably related to leaf development, while the gene *EjFT2* is involved in floral bud differentiation (Reig *et al.,* in preparation).

*Florigen* causes changes in gene expression that reprogram the shoot apical meristem (SAM) to form flowers instead of leaves. This change results in an increase of meristem mitotic activity (Bernier, 1971; Gifford and Corson, 1971). This process was studied in the annual plant *Sinapis alba*, in which the rate of cell division increased eight-fold in the central zone of the meristem and six-fold in the peripheral zone. Following the cell division and synchronization of the cells in the apex, a peak in DNA and protein synthesis was observed, this being associated with initiation of the first flower buds (Jacqmard *et al.,* 1972).

*FT* itself encodes a small globular protein of less than 20 kDa with sequence similarity to the Raf kinase inhibitor protein (RKIP) family of mammals (Kardailsky *et al.,* 1999) and with homology to phosphate ethanolamine binding protein (PEBP) (Kardailsky *et al.,* 1999; Kobayashi *et al.,* 1999). The deduced FT protein belongs to a small family of Arabidopsis proteins, which includes the TFL1 (TERMINAL FLOWERING 1) protein, whose amino acid sequence is more than 50% identical to that of FT (Bradley *et al.,* 1997) but with opposite effects on flowering. *FT* is a key target and integrator of many flowering pathways, and induction of *FT* expression leads to activation of flowering. In contrast, induction of *TFL1* results in a suppression of flowering (Bradley *et al.,* 1997; Ohshima *et al.,* 1997). Genetics analyses show that TFL1 and FT act independently in flowering control but, so far, studies in different plant species have not revealed the biochemical mechanism of this family of protein (Yoo *et al.,* 2004). For instance, a single transcription factor may be an activator or repressor, depending on which protein it interacts with (Swantek and Gergen, 2004). However, in the case of FT and TFL1, a single residue in the plant family of PEBPs is sufficient to switch signalling pathways for a whole protein (Hanzawa *et al.,* 2005).

### 1.2. Molecular events at the shoot apex: FT-FD complex and floral signalling pathways

*FT* expressed in leaves but its protein move from the inducted leaf to the shoot apical meristem (SAM) through the phloem to promote floral meristem development. In the SAM, FT protein interacts with a transcription factor of the 'bZIP' family known as FLOWERING LOCUS D (FD). FD is required for the function of FT that initiates the gene expression cascade of floral induction by inducing floral meristem identity genes such as *APETALA 1* (*AP1*), *FRUITFULL* (*FUL*) and *CAULIFLOWER* (*CAL*) genes at the SAM (Abe *et al.,* 2005; Wigge *et al.,* 2005). But flowering is regulated by several pathways in Arabidopsis that converge on the transcriptional regulation of the floral pathways integrators *FT*, *SUPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) and *LEAFY* (*LFY*) (Simpson and Dean, 2002) (Figure 1). FT and FD are interdependent partners through protein interaction and act at the shoot apex to promote floral transition and to initiate floral development through transcriptional activation of *AP1* redundantly with *LFY* (Abe *et al.,* 2005) and both are required for the induction of *SOC1* expression which is one of the earliest steps in floral induction, but it is still not know whether this is a direct or indirect effect (Abani and Coupland, 2010). In fact, induction of *SOC1* in the SAM is detected much earlier than *AP1* expression, approximately 24 h after shifting plants from short-days (SDs) to long- days (LDs) and when *SOC1* is expressed in the meristem, it interacts with *AGAMOUS-LIKE 24 (AGL24),* another MADs box transcription factor, and promotes the activation of transcription of *LFY,* which is a meristem identity gene that is involve in the initiation of floral flower development (Lee *et al.,* 2008).

Lateral meristems acquire a floral identity through the activity of the mains promoters of floral meristem-identity, *LFY* and *AP1. LFY* encodes a plant-specific transcription factor (Hamès *et al.,* 2008; Weigel *et al.,* 1992) and *AP1* encodes a MADS box transcription factor. They are expressed throughout young floral meristem and together orchestrate the switch to flower formation and early events during flower morphogenesis by altering transcriptional programs.

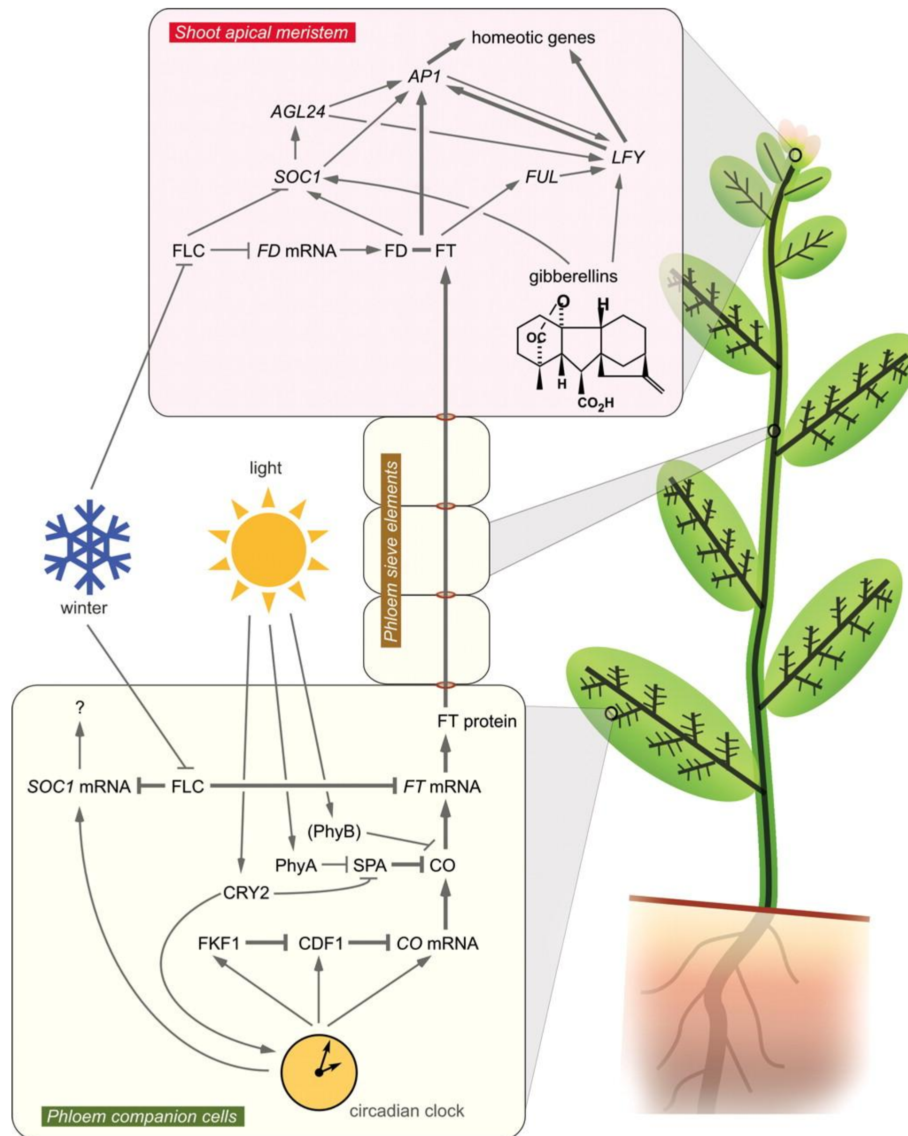


**Figure 1**. Key regulatory interactions in the leaf and at the shoot apex of A. *thaliana*. Direct interactions are indicated by bold lines (regulation of mRNA levels refers to transcriptional control). Light has a dual role in controlling the clock (for simplicity, only one line from the CRY2 photoreceptor is drawn) and CO protein stability. PhyB is shown in parentheses, to indicate that this interaction might not be cell autonomous. The schematic plant on the right illustrates the spatial relationships between the different tissues. Dark lines symbolize the vasculature. There is cellular continuity from the leaf to the shoot apex, provided by intercellular connections of plasmodesmata, shown as brown circles on the left. During the vegetative phase, the shoot apical meristem produces leaves. After flowering has been induced, it switches to the formation of flowers. (From Kobayashi and Weigel, 2007).

The close temporal sequence of *LFY* and *AP1* activation in plants implies that regulation of AP1 by LFY could be direct (Hempel *et al.,* 1997), and indeed, the LFY protein recently has been shown to bind to the *AP1* promoter (Parcy *et al.,* 1998). *AP1* can also provide feedback to activate *LFY* (Ferrandiz *et al.,* 2000). In this way *LFY* and *AP1* enhance each others expression reinforcing floral identity and initiating floral organ specification and development.

The expression of both meristem identity genes *AP1* and *LFY* is antagonized by *TERMINAL FLOWER 1 (TFL1)* preventing their ectopic expression in the centre of the shoot meristem. The main role of *TFL1* is to prevent meristem from assuming the floral identity by inhibiting the expression of *LFY* and *AP1*. Thus, in *tfl1* mutants *LFY* and *AP1* expression invades the inflorescence meristem, which are then converted into flowers (Weigel *et al.,* 1992; Bradley *et al.,* 1997). Others studies suggest that *LFY* and *AP1* prevent *TFL1* expression in floral meristem (Liljegren *et al.,* 1999; Ratcliffe *et al.,* 1999; Ferrándiz *et al.,* 2000). Besides, directly regulating *AP1* expression *LFY* also may indirectly promote *AP1* activity via negative regulation of *TFL1*. In fact, *LFY*, *AP1* and *CAL* can inhibit *TFL1* at a transcriptional level. By contrast, the final pattern of *TFL1* and floral meristem identity gene expression in the shoot apex depends on the relative timing of their upregulation, and it is maintained by distinctive mechanism of mutual inhibition.

### 1.3. The right moment to flower: flowering pathways

Plants are sessile organisms that have developed the ability to read into environmental cues and integrate them in their developmental programs to achieve this adaptation and increase their chance of survival and, ultimately, reproduction. Thus, at a certain point in their life cycle, plants undergo a major developmental transition and switch from vegetative to reproductive development. Four major pathways have been described to lead to flowering in Arabidopsis: photoperiod, vernalization, gibberellin (GA) and autonomous pathways (Blázquez *et al.*, 2006; Wilkie *et al.,* 2008). Photoperiod and gibberellin are promoting pathways since they regulate the expression of genes that cause the floral transition in this plant model. These have been called 'floral pathway integrators' and include *FT*, *LFY*, and *SOC1* (Nilsson *et al.,* 1998;

Kardailsky *et al.,* 1999; Kobayashi *et al.,* 1999; Blázquez and Weigel, 2000; *Lee et al.*, 2000; Samach *et al.,* 2000). Recent work has revealed the existence of a separate floral pathway involving light quality closely connected to ambient temperature (Blazquez *et al.,* 2003). By contrast, the pathways that enable the floral transition regulate the expression of floral repressors, such as vernalization and autonomous pathway (Figure 2) that regulate the floral repressor *FLOWERING LOCUS C* (*FLC*). High levels of the floral repressors keep the meristem 'blind' to promotive floral signal.

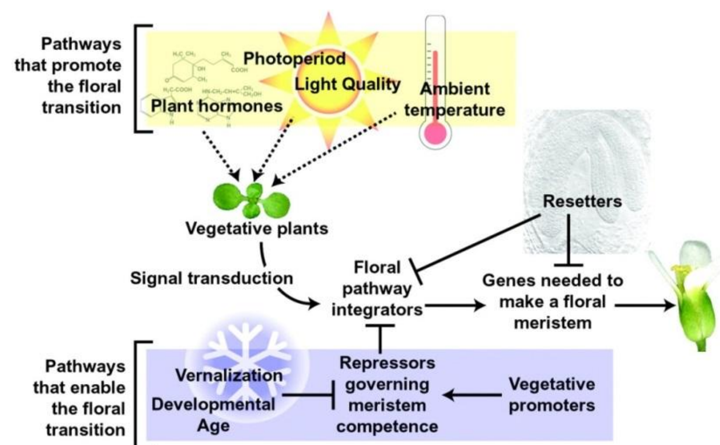


**Figure 2.** Pathways That Enable or Promote the Floral Transition Determine Flowering Time.The different pathways are grouped into those that promote and those that enable the floral transition. (From Paul K. Boss *et al.* Plant Cell 2004).

### 1.3.1. Pathways that promote floral transition

#### 1.3.1.1. Photoperiodic pathway

The most consistent model explaining the mechanisms by which photoperiod information is integrated into the regulation of development in Arabidopsis is 'the external coincidence model' (Yanovsky and Kay, 2003; Putterill *et al.,* 2004). This model propose that an endogenous oscillator regulates rhythms with a period of ~24 h (circadian clock) in combination with signals from photoreceptors to sense day length. The crucial aspects of the day-length measurement mechanism are the circadian regulation of *CONSTANS* (*CO*) gene expression and the light-regulation of CO protein stability and activity (Hayama and Coupland, 2004). *CO* mRNA expression is regulated by the

circadian clock and photoperiod, and under short-day (SD) peaks in the night and under long-day (LD) peaks toward the end of the day (Suarez-Lopez *et al.,* 2001) an expression profile that is mirrored by *FT*. The *CO* gene encodes a B-box-type zinc finger transcriptional activator that induces the expression of *FT* gene in a light-dependent manner (Yanovsky and Kay, 2002). In addition, both genes show distinct tissues specific expression patterns in which *CO* is expressed in both leaf and stem phloem, and *FT* is expressed only in the leaf phloem (Takada and Goto, 2003).

Despite these advances in understanding the photoperiod regulation of flowering, the mechanism that generates the daylength-dependent expression profiles of CO remains unclear.

#### 1.3.1.2. GA pathway

Genetic studies have confirmed the physiology findings that gibberelins also actively promotes flowering in Arabidopsis (Langridge, 1957). Conversely, a decrease in GA levels or insensitivity to GA signaling, delays flowering although this effect is significantly only in SD (Wilson *et al.,* 1992).

Double mutant analysis have established that the GA pathway is genetically distinct from the photoperiod promoting pathway and have confirmed that GA pathway has less influence on flowering time in LD than in SD (Reeves and Coupland, 2001). However, in the absence of LD promotion pathway, the GA pathway is an important promoter of flowering.

One target of the GA signal is *LFY*, because *LFY* promoter activity is increased by exogenous GA application (Blazquez *et al.,* 1998), so GAs may alter flowering time by increasing *LFY* expression. Constitutive expression of *LFY* accelerates flowering but cannot fully complement the flowering time (Blazquez *et al.,* 1998). This may be attributable to another floral integrator, *SOC1*, also being regulated by GAs (Moon *et al.,* 2003a). In fact, $GA_4$, which is most likely produced in the leaves and transported to the meristem, up-regulates one or both of the genes *LFY* and *SOC1* leading to flowering.

### 1.3.2. Pathways that enable the floral transition

#### 1.3.2.1. Vernalization pathway

Vernalization is the process by which prolonged exposure to cold renders plants competent to flower (Chouard, 1960). Plants that require vernalization to flower encode repressors that block flowering during summer or autumn until the plant is exposed to low winter temperatures. In fact, vernalization strongly downregulates *FLC* levels accelerating flowering (Sheldon *et al.,* 1999) (Figure 3). The cold signal is perceived in the shoot apical meristem many days before the meristem transition by activation of the cold-response genes *VERNALISATION 1 (VRN1), VRN2* and *VRN3* and by changes in DNA methylation (Finnegan *et al.,* 1998). These findings suggested that vernalization had an epigenetic basis (Wellensiek, 1962). The main function of *VRN1* and *VRN2* is to maintain *FLC* repression suggesting that they are part of the cellular machinery that provides a memory of vernalization whereas that *VERNALIZATION INSENSITIVE3* (*VIN3)* is involved in the establishment of the repression of *FLC* (Sung and Amasino, 2004). The reduction in *FLC* expression in low temperatures involves expression of an antisense RNA (Swiezewski *et al.,* 2009) and machinery that modifies the tails of histone 3 at the *FLC* gene, particularly trimethylation of lysine 27 (Finnegan and Dennis, 2007). These processes result in the diminished *FLC* mRNA expression in the cold and stable repression of *FLC* when plants are returned to normal growth temperatures. Moreover these changes in *FLC* chromatin are dependent on mutations in the vernalization pathway (Bastow *et al.,* 2004). But *FLC* is not the only flowering time target of vernalization. Like *SOC1, AGL24* is also regulated by vernalization, but unlike *SOC1*, this upregulation is independent of *FLC* activity. *AGL24* appears to act downstream of *SOC1* but upstream of *LFY*, but overexpression of *AGL24* increases *SOC1* expression, indicating that the relationship between these genes may not be straightforward (Michaels *et al.,* 2003a). Additionally, the MADS box transcription factors *FLC* are expressed in the leave where they repress *FT* and *TWIN SISTER OF FT (TSF)* and in the SAM where act as direct repressors of *SOC1* (Lee and Lee, 2010; Searle *et al.,* 2006; Lee *et al.,* 2007; Jang *et al.,* 2009; Liu *et al.,* 2009). *FLC* directly bind to

*FT* and blocks its transcription until the plant is exposed to low temperatures that repress *FLC* transcription, allowing for the induction of *FT* the following spring as the photoperiod lengthens.

#### 1.3.2.2. Autonomous pathway

The autonomous pathway comprises a combination of factors involved in RNA processing and epigenetic regulation that downregulate the floral repressor, *FLC.* Moreover, the genes of the autonomous pathway do not function in a linear hierarchy but as a series of subgroups sharing a common function; reduce *FLC* mRNA accumulation (Sheldon *et al.,* 2000b). *FLC* antagonises the activity of pathways that promote flowering in response to day-length and the phytohormone gibberellic acid by repressing the set of target genes that is activated by these pathways (Reeves and Coupland 2001). Thus autonomous pathways genes promote flowering indirectly by repressing *FLC*.

*fca, fld, fpa, fve, fy, ld, flk* and *ref6* mutants are classified as functioning in an autonomous pathway because they flower late in all photoperiods (Lee *et al.,* 1994). Two other genes, *LUMINIDEPENDENS* (*LD*) and *FLOWERING LOCUS D* (*FLD*) are negative regulators of FLC that show genotype-dependent effects on flowering time (Sanda and Amasino, 1996).

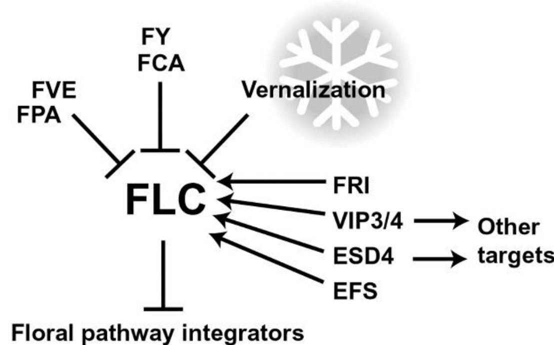


**Figure 3.** Pathways That Enable the Floral Transition.A central regulator of the enabling pathways is the floral repressor FLC. High levels of FLC repress the activity of the floral pathway integrators, and this antagonizes their activation by floral promotion pathways. (From Paul K. Boss *et al*. Plant Cell 2004).

In a similar manner as the rest of the genes involved, *FLD* and *LD* act to deacetylate *FLC* chromatin, preventing the transcription of *FLC* and promoting flowering (He *et al.,* 2003). Thus, epigenetic regulation via chromatin modification is emerging as a major mechanism to modulate *FLC* levels.

## 2. Flowering in woody perennial plants

Flowering is an integral developmental process in angiosperms, crucial to reproductive success and continuity of the species through time. Some angiosperms complete their life cycle within a year (annual plants), and others have a longer reproductive life, which is characterized by the generation of new flowering and vegetative shoots every year (perennial plants). Despite the differences in their lifespan, the underlying genetics of flower induction and floral organ formation appears to be similar among these plants. Hence, the knowledge gained from the study of flowering mechanism in *Arabidopsis thaliana* can be used to better understand similar processes in other plant species, especially the perennials, which usually have a long generation time and are not amenable to genetic analysis. Using Arabidopsis as a model, we briefly discuss the current understanding of the transition from vegetative to reproductive growth and the subsequent formation of individual floral organs, and how this knowledge has been successfully applied to the identification of homologous genes from perennial crops. Although annuals appear to share many similarities with perennials in terms of gene function, they differ in their commitment to flowering. Once an annual reaches the reproductive phase, all meristems are typically converted into either floral or inflorescence meristems. In contrast, each year, each meristem of a mature perennial has the choice to produce either a vegetative or a reproductive shoot. Using the DNA sequence of flowering genes from model plants as a starting point, flowering genes have been successfully isolated from several agriculturally important tree crops, including apples (Yao *et al.,* 1999; Sung *et al.,* 1999; Kotoda *et al.,* 2000; Wada *et al.,* 2002), *Citrus* (Pillitteri *et al.,* 2004a; Pillitteri *et al.,* 2004b; Endo *et al.,* 2005), grapes (Boss *et al.,* 2001; Boss *et al.,* 2002; Boss *et al.,* 2006), loquat (Lin *et al.,* 2006; Liu *et al.,* 2010, 2013) eucalypts (Kyozuka *et al.,* 1997; Southerton *et al.,* 1998; Dornelas *et al.,* 2004), and other perennials of

commercial importance (Dornelas *et al.,* 2005, 2006; Esumi *et al.,* 2005; Qingyi *et al.,* 2005).

Woody perennials plants, apart from responding to positive environmental signals, are also sensitive to adverse conditions, such as water, nutrient deficiency and heat. Under such conditions, different strategies are employed in order to survive these potentially life- threatening situations. One of the most obvious responses is profuse, out of season blooming. Nevertheless, for woody perennial species, the developmental stage can influence the timing of flower initiation which has distinguishable juvenile and adult phases. However, unlike in many annuals, the conversion to flowering apices only occurs in some meristem, whereas others maintain their indeterminate vegetative state (Jackson and Sweet, 1972), essential for continued growth of the plant. But after becoming reproductive, perennial crops maintain a proportion of vegetative meristems to allow further vegetative growth over multiple seasons. Consequently, much of the research on the flowering physiology of woody perennials has been concerned with seasonal changes in the flowering behavior, and this contrasts with the research using annual plants, which has focused on the transition from vegetative to reproductive development.
Flowering in higher plants is a complex event involving tightly regulated interactions between external and internal factors which include the genes that regulate flower initiation and development. Studies are ongoing to determinate the endogenous functions of these genes, which in turn will provide the molecular framework for elucidating the underlying mechanism of flowering in woody perennials. In this regard, one of the key issues is to determine if buds on mature perennials plants, that do not subsequently produce flowers, can be considered to be ‘vegetative’ or are in fact ‘reproductive’ buds that failed to produce visible flowers because of physiological constraints, or ‘floral reversion’, during bud development prior to shoot growth.

### 2.1. Flowering in woody Rosaceae fruit trees

The Rosaceae family includes several economically important subfamilies. Amygdaloideae contains the genus *Prunus*, which includes stone fruits species such as peach (*P. persica*), plum (*P. domestica*), and sweet cherry (*P. avium*).

Also in Amygdaloideae are other genus like *Malus*, *Pyrus* and *Eriobotrya* (Potter *et al.,* 2007; McNeill *et al.,* 2012).

These species are perennial woody plants which are adapted to temperate climates and typically bloom in early spring from flower buds developed during previous growing season (Grainger, 1939; Wilkie *et al.,* 2008), but each species has specific features in its yearly growth cycle. In fact, there are important differences in flowering patterns between deciduous and evergreen species. Generally, floral induction in adult deciduous trees is initiated during summer and flower buds develop just after floral induction, during fall, short days induce growth cessation and bud set, after which, the tree enters dormancy and bud growth restarts with the onset of spring. In contrast, in evergreen fruit trees species, like some *citrus spp*, low temperature during fall and winter is the most important factor leading to floral induction (Krajewski and Rabe, 1995) except in genus *Eriobotrya.* Thus, the season of floral induction and flower bud development may differ markedly between evergreen and deciduous trees (Figure 4), indicating that the factor triggering annual floral induction and differentiation may also differ between these two groups.

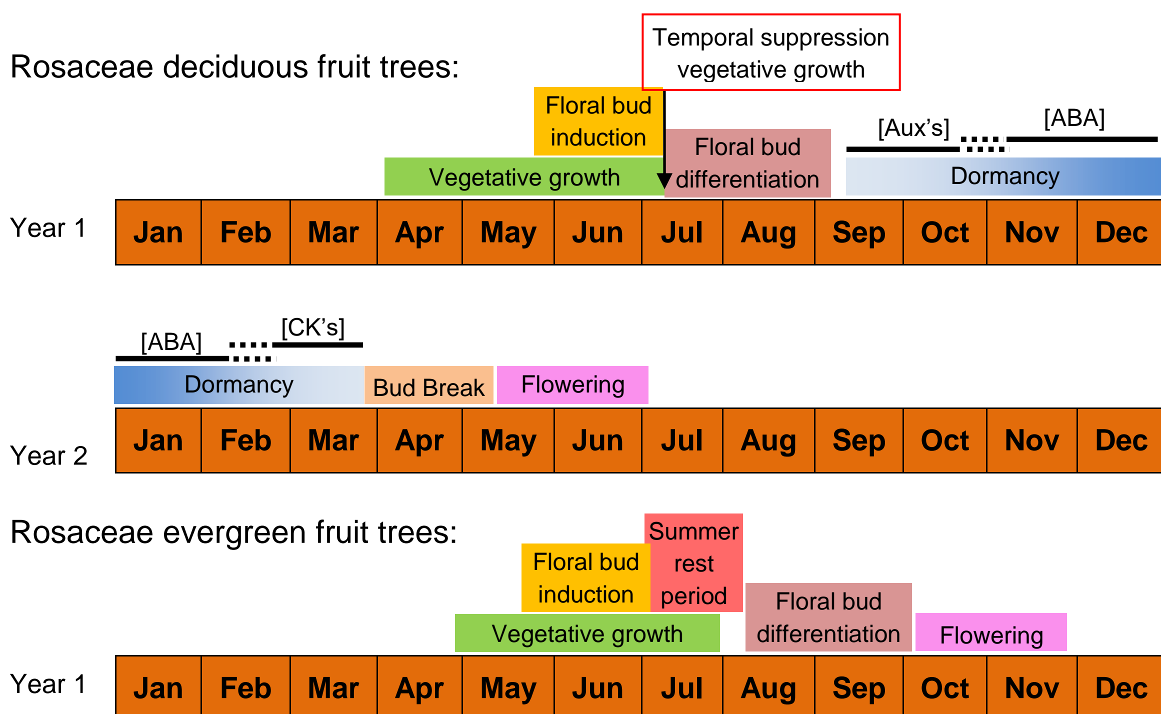


**Figure 4.** Diagrammatic representation of the annual growth cycles of Rosaceae deciduous and evergreen fruit trees.

Recently, homologues of genes involved in the Arabidopsis flowering process appear to play a key role in seasonal woody species (Hsu *et al.,* 2006; Nishikawa *et al.,* 2007). In apple, which is deciduous, the transcription of homologues of *LFY* and *AP1*, which act as floral activators, is correlated positively with floral bud development during summer, whereas the transcription of *TFL1*, which is a floral repressor, is correlated negatively with flowering season (Kotoda and Wada, 2005). Accordingly, in early summer, when flower initiation occurs, *MdTFL1* is no longer detectable, and the expression levels of *MdAP1*, *MdLFY* and *MdFT* are upregulated in the terminal shoot apex (Mimida *et al.,* 2011a). At the same time, the SAM ceases to produce new leaf primordia, shoot growth stops, and floral differentiation begins. Flowering induction in apple have been proposed to occur at the end of May, just before histological and morphological changes can be detected in the meristem (Hattasch *et al.*, 2008). Spring bloom in apple is part of the cycle of reproductive development that begins with floral initiation in the preceding summer. Flowers are produced on terminal inflorescences of spurs (short shoots) or long shoots. The process is not continuous, but broken by winter dormancy, a period of rest associated with the winter months, during which no development occurs, and flower buds increase in weight and enters maturation (growth of flower organs and development of flowers) last until the following spring when blooming take place.

In contrast to other deciduous Rosaceae species, the reproductive development of loquat, an evergreen Rosaceae species is a continuous process that is not interrupted by periods 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 (Lin *et al.,* 1999).

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). In loquat, whereas one-year-old leaf removal assays revealed that flower induction takes place from late in May to early in June (Fatta del Bosco, 1961), microscopic studies reveal the first indications of flower commitment in late July (Rodriguez *et al.,* 2007). Accordingly, the transcripts of *EjLFY* were detected in floral shoot apexes during this period until

the flowers formed. Although similar dates for flowering induction and flowering initiation have been reported in other Rosaceae fruit bearing trees, such as apple (Foster *et al.,* 2003) or sweet cherry and peach (Engin and Unal, 2007) loquat presents an important feature. Whereas in these other temperate deciduous trees the flowering development extends over two consecutive seasons with a rest period in between, loquat is able to complete flower induction, initiation and differentiation in only one season. 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.

## 3. Environmental factors regulating flowering in woody species

Flowering is an integral development process in angiosperms, crucial to reproductive success and continuity of the species through time. Perennials plants have a long reproductive life which is characterized by the generation of new flowering and vegetative shoots every year once they reach the reproductive phase. This process is often triggered by environmental cues and coordinated by internal factors. By tailoring their reproductive plan to favorable environmental conditions, plants can ensure the success of their progeny. This is especially important for those growing in regions with seasonal changes especially in day length and temperature. The ability to detect seasonal changes and respond to it confers a selective advantage to plants because it provides a means of anticipating and change their growth in accordance with climate, being flowering process in part a seasonal phenomenon.

### 3.1. Photoperiodic control of flowering

One of the most important external factors controlling flowering time in temperate include day length (in fact the length of the night). This regulatory effect of day length on the initiation of flowering was first described by Garner and Allard more than 80 years ago. In fact, they discovered that shortening day length induced flowering in Maryland Mammoth tobacco plants (Garner and Allard, 1920). They also explored day-length-dependent flowering responses in other plant species and they classified plants per photoperiodic responses into

LD plants, in which flowering occurs when the day becomes longer than some crucial length, SD plants, in which flowering occurs when the day becomes shorter, and day-neutral plants whose flowering regime is independent of the duration of light exposure. Depending on species, cultivars and geographic locations, plants can respond to changing light and dark period by adjusting their flowering time to be in tune with their surroundings. This is related by the circadian clock which has been shown to modulate photoperiodic responses in addition to regulating other physiological events such as the daily rhythms of leaf movement and the opening and closing of stomata (Samach and Coupland, 2000, Mizoguchi *et al.,* 2005). In fact, whereas photoperiod induction is a common mechanism in herbaceous species and constitutes a well understood genetic flowering pathway, in fruit trees is not well understood, since they are generally considered as day-neutral plants (Wilkie *et al.,* 2008). However, careful analysis in controlled conditions should be carried out, since a few studies suggest that photoperiod may affect flower induction in apples (Cain 1971), avocado (Buttrose and Alexader, 1978), kiwi (Grant and Ryugo, 1984), and olive (Stutte and Martin, 1986), although it is most likely not an inductive stimulus but a secondary factor. In fact, *Prunus* species show a pronounced photoperiod/temperature interaction in the control of growth cessation and dormancy (Heide, 2008) and apple cv Jonat have increased flower bud formation when photoperiod increased from 8h to 12 or 14h (Stahly and Piringer, 1962).

### 3.2. Temperature control of flowering

The influence of ambient temperature of flowering appeared even though small fluctuations in temperature have dramatic effects on flowering time. Recent advances in Arabidopsis have uncovered multiple molecular mechanisms controlling ambient temperature regulation of flowering, which modulates both repressing and activating factors of flowering time at lower and higher ambient temperatures, respectively. Despite the substantial progress that has been made in understanding the molecular mechanisms, it remains unclear how temperatures are sensed by plants. It is imaginable that there is a direct physical

effect of higher temperature on the folding molecular interactions, and stability of the chromatin, mRNA and protein involved (Nocker *et al.,* 2001).

Further research is needed to understand the thermometer of flowering time control in plants, since at time temperature is rising worldwide and that temperature signals can be crucial for crop production. In fact, the temperature effect is dominant over photoperiod and sometimes flower induction only occurs within a certain permissive temperature range. Additionally, long exposure to chilling contributes to seasonality of flowering, since it changes the physiological status of the plants so that further flower initiation is prevented ever under an inductive photoperiod in the spring (Guttridge, 1985). Prolonged exposure to cold temperatures (vernalization) constitutes a flowering pathway in Arabidopsis, although it can also affect many other physiological processes. Recent work has linked a family of Helix-loop-Helix transcription factor *PIF (PHYTOCHROME INTERACTING FACTOR)* to regulation of flowering time in Arabidopsis. PIF4 modulates thermal induction of flowering by directly binding to and activating expression from the FT promoter (Kumar *et al.,* 2012). Chromatin immunoprecipitation (ChIP) analysis of *PIF4* at a range of temperatures has confirmed that the ability of *PIF4* to bind to the *FT* promoter is dependent on temperature, suggesting a direct mechanism by which temperature can modulate the ability of *PIF4* to bind its targets. Otherwise, the circadian clock control *PIF4* and *PIF5* expression (Nozue *et al.,* 2007, Dixon *et al.,* 2011) and both act together to match floral development to the light and temperature environment. Thus, both proteins are implicated in a signalling pathway that stimulates *FT* expression in a largely *CO*-independent manner. Therefore, in fruit trees, temperature also plays an important role in physiological processes like flowering since many species require and exposure to periods of low or temperate temperatures for flowering to be promoted (Hay and Ellis, 1998). The temperature treatment is required to remove floral repressors (Andersen *et al.,* 2004). This mechanism appears to ensure that flowering, particularly for plants growing at high latitudes, will not occur until spring or early summer, when longer days coupled with higher temperatures favour floral bloom but it is not clear in woody perennials which in temperate regions, meristem fall dormant in winter, as an adaptive strategy to protect sensitive growing tissue form

unfavourable conditions (Jones *et al.,* 2013; Luedeling *et al.,* 2012). During winter dormancy, the meristem has the inability to resume growth under favorable conditions (Rohde and Bhalerao, 2007).

Meristem growth cessation and dormancy induction by low temperature seems to be rather widespread within the Rosaceae. However, experiments done in *Prunus* species demonstrate a pronounced photoperiod/temperature interaction and temperature in the regulation of growth cessation and dormancy (Heide, 2008).

Low temperatures also provide as an inductive signal in several subtropical and tropical tree species like mango (Whiley, 1992), lychee (Menzel and Simpson, 1995), macadamia (Nakata, 1976), avocado (Buttrose and Alexander, 1978) and orange (Moss, 1976). Low temperature during winter also induce flowering in olive, an evergreen tree species adapted to temperate conditions (Hackett and Hartmann, 1964). Nevertheless, in temperate deciduous trees, temperature can affect the intensity of floral initiation, but it is not clear if it provides an inductive stimulus.

### 3.2.1. Chilling and heat requirement in fruit trees

In temperate-zone deciduous fruit trees, temperatures or day lengths below a certain threshold impede the processes that lead to growth and prevent any external indication of activity like dormancy and bud break necessary for a successful flowering. In woody species, cycling between dormancy, and therefore growth, must be synchronized with the seasonal climatic variations. Many efforts have been made to model plant responses and phenology to temperatures being Reaumur (1735) first introduced the concept of degree-day summation. He suggested that differences between years and locations in the date of phenological events could be explained by differences in daily temperatures from an arbitrary date to the date of the phenological event considered. Despite initial disagreement regarding the temperature threshold for dormancy breaking (7.2°C) was finally adapted (Samish, 1954; Vegis, 1964). By approximation, 7°C was adopted as the useful temperature threshold with respect to overcoming endodormancy. Concomitantly, the concept of chilling

requirement (CR) was established (Samish and Lavee, 1962) being influenced by cultivar and conditionated by year to year variation (Saure, 1985). In addition, crop specific models for CR and heat requirement (HR) and/or flowering process have recently been developed as more specific approach in different countries and species like olive in Spain and Portugal (De Melo-Abreu *et al.,* 2004), apple in Italy (Rea and Eccel, 2006), pear in Portugal (De Melo-Abreu *et al.,* 2005) and kiwifruit in New Zealand (Austin *et al.,* 2002). They include simple heat units based on the accumulation of daily mean temperatures above a certain threshold temperature during growing period (Wiggans 1956, Brown 1960, Wang 1960, Baskerville and Emin 1969, Chen 1973). Nevertheless, it is still not clear whether cultivars have specific HRs for flowering (Alonso *et al.,* 2005) or whether flowering date is determined basically by CRs (Okie and Blackburn, 2011). Since dormancy breaking is progressive and might be the result of diverse combinations of chill and heat. When supra-optimal chill is supplied, the need for heat is lower. Moreover, several studies have reported that the onset of flowering begins at the end of the summer, prior to the arrival of chilling temperatures (Hauagge and Cummins, 1991b) even though summer temperature could be relate to the intensity of dormancy (Chuine and Cour, 1999). Therefore, it was soon perceived that CRs tended to be highly variable and dependent on the year and location called into question its consistency and suitability for measuring the quantity of cold required to overcome dormancy over a period.

It is commonly assumed that chilling and forcing requirements are fulfilled sequentially, with heat only being effective after sufficient chill has accumulated (Cannell and Smith 1983; Cesaraccio *et al.,* 2004; Fuchigami and Nee 1987; Rea and Eccel 2006). However, parallel models with overlapping chilling and forcing phases have also been suggested (Hänninen 1990, 1987; Kramer 1994). The decision about which type of model to use is often made a priori in phenology modeling studies, and models are then fitted to available data. Numerous proposals have been made, indicating the notable difficulties associated with delimiting this phenomenon. Three chilling models are used widely around the world: the Chilling Hours Model (Weinberger 1950), the Utah Model (Richardson *et al.,* 1974), and the Dynamic Model (Fishman *et al.,* 1987).

The Utah Model assigned chill units values to different temperature ranges. Subsequently, models adjusted regarding this were developed as the low chilling Model (Gilreath and Buchanan, 1981b). The Dynamic Model was developed for the warm winters and is thought to be a milestone in dormancy modeling assuming that chill accumulates by a two-step process. The first is de accumulation of an intermediate product promoted by cold temperature and reversed by warm temperature. However, once a sufficient amount of the intermediate product has accumulated, Chill Portions are permanently accumulated. Methods for determining the heat requirement for blooming have been also developed (Anderson *et al.,* 1986 and Richardson *et al.,* 1974). These methods essentially consist of establishing the heat accumulation above a threshold, to which a tree is exposed from dormancy breaking until flowering. The Growing Degree Hour Model (Anderson *et al.,* 1986; Luedeling *et al.,* 2009) has been used widely as a forcing model. However, much more research is needed to develop or identify the most appropriate model.

## 4. Plant hormones regulating flowering

Plant hormones are most prominent among long-distance-signals and almost all of them are already extensively used by the plant to interfere with floral bud induction and other physiological processes such as vegetative growth, apical dominance, assimilate partitioning, fruit set and growth, or stress situations (Hoad *et al.,* 1993; Bangerth, 2000). Their effect in the control of flowering time depends on the specie.

**Gibberellins** (GAs) are strongly associated with flowering. Genetic studies have confirmed that GAs accelerate Arabidopsis flowering (Langridge, 1957). Plants overexpressing GA-20 oxidase, a gene late in the GA biosynthesis pathway (Figure 5), are early flowering in both long days and short days (Huang *et al.,* 1998; Coles *et al.,* 1999) and exogenous applications also accelerates flowering in wild-type Arabidopsis, particularly in short days (Langridge, 1957). In fact, GA application can even substitute for a vernalization treatment (Sheldon *et al.,* 1999). There is a causal connection between endogenous GA levels and flowering in Arabidopsis confirmed by signaling mutants. In some species of

gymnosperm trees, applied low polar GAs are also able to induce precocious and prolific floral induction (Pharis and King, 1985). Conversely, a decrease in GA levels or insensitivity to GA signalling, delays flowering although only in SD (Wilson *et al.,* 1992). For example, a mutation in $GA_1$, the first committed step in GA biosynthesis, makes Arabidopsis an obligate LD plant because it no longer flowers under SD. How changes in GA biosynthesis or signal transduction result in altered flowering time is an area of active research.

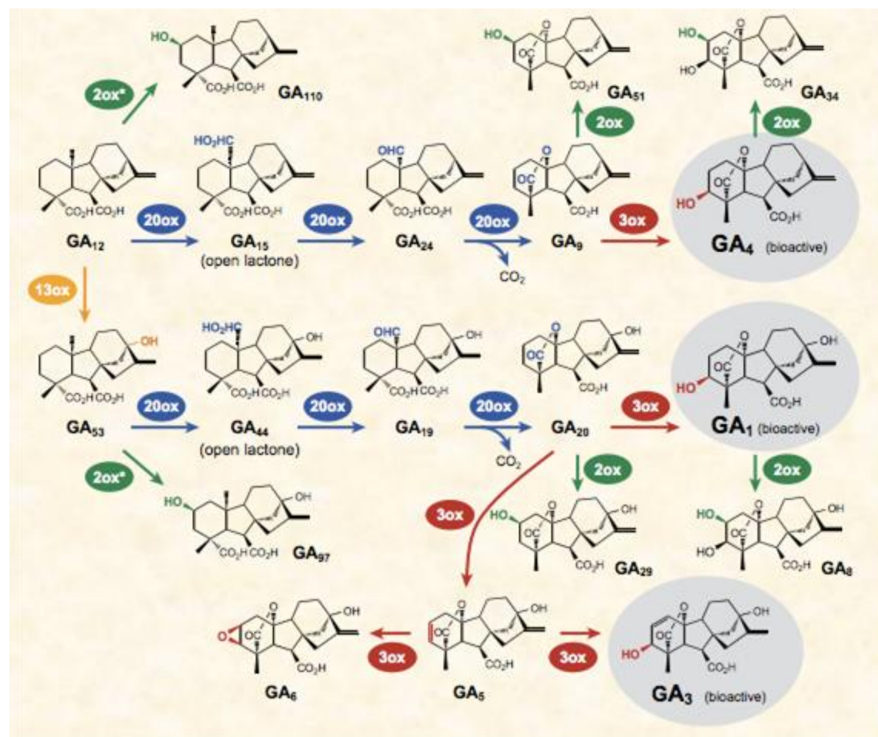


**Figure 5**. GA biosynthesis pathway. (Adapted from Yamaguchi, 2008)

However, in most angiosperm trees, GAs have the opposite effect. Luckwill (1970) in pome fruits hypothesized that gibberellins produced in seeds inhibit flower formation since they coincide with embryo growth in developing fruitlets. Accordingly, for many polycarpic plants, the application of gibberellic acid ($GA_3$) during flower induction interrupts the floral process and partially reduces the intensity of flowering. $GA_3$ applications downregulated *CiFT* expression in leaves of sweet orange (Muñoz-Fambuena *et al.,* 2012). This technique has been used efficiently in pome fruits (Tromp, 2000), mangoes (Turnbull *et al.,* 1996), avocados (Salazar-García and Lovatt, 2000), apricots (Southwick *et al.,* 1997), *Citrus* (Monselise and Halevy, 1964), peaches and nectarines (Painter and Stembridge, 1972) and plums (González-Rossia *et al.,* 2006).

Particularly, in loquat gibberellins applications during the floral bud induction and floral bud differentiation periods significantly reduced flowering intensity because of a reduction in the number of premature flowering shoots. Otherwise, $GA_3$ application directly in the apex, during the floral bud differentiation period, reduced the number of flowers per panicle but not avoid the apex differentiation (Reig *et al.,* 2011).

**Indole acetic acid** (IAA) is the only plant hormone with a strictly polar and highly-regulated transport pathway (Muday and DeLong, 2001). Signals are independent of sink. Further, by some kind of 'auxin transport auto-inhibition', this hormone transport is able to affect a particular organ, for example, a bud/meristem, without entering it (Bangerth, 2000). The most prominent example of this is apical dominance. Bangerth (2009) suggested that polar IAA transport, a long-distance signal, could act as a secondary messenger to GAs in floral bud induction. In fact, the application of gibberellins to apple trees considerably stimulated IAA-export of fruit and shoot tips which would be in line with a second messenger role for IAA (Callejas and Bangerth, 1997). Concomitantly, triiodobenzoic acid (TIBA) applications, a class of IAA-transport inhibitor, stimulated the floral bud induction in some fruit (Luckwill, 1969; Ito *et al.,* 2001; Bangerth, 2009) and thus several horticultural procedures reducing polar IAA transport are used to increase floral bud induction (Blaikie *et al.,* 2004). Lastly, application of an auxin to a decapitated shoot tip is also reported to inhibit floral induction (Tamim, 1996).

**Cytokinins** (CKs) applications promote bud floral induction in monocarpic and polycarpic plants in contrast to GAs and IAA transport (Ramirez and Hoad, 1981; Bernier and Périlleux, 2005) but not in a low concentration since transgenic CK-deficient Arabidopsis plants never flowered (Werner *et al.,* 2003). At medium CK concentrations, floral bud induction occurred, but high concentrations promoted only vegetative development in *Sinapis alba* (Bernier and Périlleux, 2005), *Vitis vinifera* (Srinivasan and Mullins, 1981) and *Malus domestica* (Bangerth, 2009). Despite the large amount of physiological evidence implicating cytokinins in flowering time control (Bernier *et al.*, 1993), genetic evidence for a role for cytokinins in the promotion of Arabidopsis flowering is

lacking. Other experiments focusing on the effect of water stress increasing the CKs concentration of xylem, found a concomitant quantitative increase in floral bud induction (Stern *et al.,* 2003), but the application of other, non-CK compounds, like maleic-hydrazide and TIBA, had also significantly increased floral bud induction of apple, pear, olive and mango trees (Luckwill, 1970; Ben-Tal and Lavee, 1985; Ito *et al.,* 2001; Blaikie *et al.,* 2004). Both maleic-hydrazide and TIBA are potential inhibitors of the IAA polar transport and/or metabolism, so that the CK increase may be the cause for the increased floral induction rather than the IAA transport (Bangerth, 2000).

**Abscisic acid** (ABA) it is also related to flowering since it generally considered as an inhibitor of flowering, although in some experiments it appeared to be a promoter. Mutant of Arabidopsis that reduce ABA biosynthesis are earlier flowering under non-inductive conditions, suggesting that ABA inhibits flowering (Martinez-Zapater *et al*., 1994) In support of this, abi1 and abi2 (abscisic acid signalling mutant) have been shown to reduce the flowering time (Chandler *et al.,* 2000). Moreover, exogenous ABA applications inhibit flowering in Arabidopsis and other plants including darnel (*Lolium temulentum*), (Blazquez *et al.,* 1998; Domagalska *et al.,* 2010; King and Evans 1977) via the downregulation of *FT*. In fact, independent of the well-established endogenous ABA signalling, ABA applications to the whole plant thus negatively regulated the *FT* expression. One hypothesis is that ABA applications in Arabidopsis might be hampered by the intrinsic spatial and temporal-dependent regulation of ABA signalling, thus making it difficult to distinguish which experimental design fully mimics endogenous ABA action (Conti *et al.,* 2014). The location and timing of ABA application could play a crucial role in determining how ABA affects flowering. Nevertheless, endogenous ABA also acts as a positive regulator of the drought escape (DE) response via the upregulation of the key floral gene *FLOWERING LOCUS T* (*FT*) (Riboni *et al.,* 2013), activating flowering genes. Drought stress results in an increase in *FT* expression without affecting the physiological circadian oscillation of *FT* (Riboni *et al.,* 2013; Su *et al.,* 2013). Because the pattern of FT transcript accumulation is mainly dictated by variations in CO protein levels, ABA might directly affect CO protein levels and/or activity. Since ABA accumulates primarily in the vascular tissue (Endo *et*

*al.,* 2008; Koiwai *et al.,* 2004; Cheng *et al.,* 2002), overlapping with the site of *FT* expression, a further possibility could be that ABA promotes *GI (GIGANTEA)* activity, independent of CO, by facilitating its direct action on *FT* promoter. ABA also could mediate several endogenous and environmental stimuli that affect flowering via regulation of *FT* levels such as is warm ambient temperature which causes the upregulation of several ABA-related transcripts and increase endogenous ABA levels (Balasubramanian *et al.,* 2006; Toh *et al.,* 2008) and upregulate *FT* expression independent of CO (Kumar *et al.,* 2012). These correlative observations may point to a role for ABA in warm ambient temperature-mediated flowering response.

Shalom *et al.* (2014) identified a transport of ABA from fruit to buds in citrus, being the ABA content higher in buds of ON trees whereas the *NCED3* genes, which determine synthesis of ABA, presented a higher expression in buds of OFF trees suggesting that the content of ABA in ON buds is produced in other part of the tree, particularly, the fruit (Shalom *et al.,* 2014). Concomitantly, exogenous ABA treatment to buds of *Citrus unshiu* reduced sprouting and flowering (García-Luis *et al.,* 1986). Nevertheless, in stress conditions ABA content in leaves (Gómez-Cadenas *et al.,* 2000; Koshita and Takahara, 2004) and *CiFT* expression (Chica and Albrigo, 2013) increased conciding with flowering promotion (Koshita and Takahara, 2004). ABA is mainly known as a stress hormone; however, the last experiment suggests that ABA might be an endogenous component affecting the floral transition. In fact, the ABA level gradually increased in buds and adjacent leaves in apple during the flower induction process (Xing *et al.,* 2015). In litchi whereas control trees and ABA-treated trees had a similar percentage of flowering terminal shoots, the number of flowers per panicle in the latter was significantly higher than that of the upper, suggesting that ABA promoted flowering (Cui *et al.,* 2013).

## 5. Hypothesis and objectives

Rosaceae deciduous fruit trees grown in climates with well- differentiated seasons fall dormant every year to survive unfavourable conditions during the winter. Cycling between dormancy and growth finely synchronised with seasonal climatic variations. CRs and HRs are needed to overcome dormancy and to flower, respectively, but dormancy breaking is progressive and the result of the chill and heat combination. In contrast, in Rosaceae evergreen fruit trees, such a loquat, a period of chilling accumulation during winter is not required. Nevertheless, in both, floral bud induction and floral bud differentiation processes coincides on time, during late spring and in summer, respectively, after a period of slow-down in growth for the second one, influenced by high temperatures.

Since recent data indicates that low temperature control growth cessation and dormancy induction (Cook *et al.,* 2005), Chuine and Cour (1999) also documented that summer temperatures can also be related to control growth cessation and the intensity of dormancy.

The hypothesis tested in this PhD thesis was:

Growth cessation caused by high summer temperature is required for floral bud differentiation.

To prove this hypothesis the following objectives were established:

1. To study the effect of terminal apex development on the flowering of lateral shoots.

2. To study the effect of summer temperature on floral bud differentiation by growing trees indoors at 25ºC average maximum temperature.

3. To develop a specific model to establish the heat accumulation needed during summer for flower bud differentiation.

4. To quantify the endogenous content of plant hormones (GAs, ABA, AXs and CKs) in shoot apex during the flower bud differentiation period.

5. To characterize the expression of the genes related to the floral transition (*EjLFY*, *EjAP1* and *EjTFL1*) in shoot apex during the flower bud differentiation period.

6. To observe the anatomical characteristics of flower bud differentiation and the differences in the time.

# *Materials and methods*

# 1. Plant material and experimental design

## 1.1 Section 1. Determination of the effect of current shoot apex removal on floral bud differentiation of premature shoots

This experiment was conducted in 2014, 2015 and 2016 on adult ‘Algerie’ and ‘San Filippara’ loquat trees *Eriobotrya japonica* (Lind.) grafted onto loquat seedling and grown in three commercial growing areas, Callosa, Alicante, Spain (38°39′N; 00°07′W), Palermo, Sicily, Italy (38°04′N; 13°25′E) and Valencia, Spain (39º 29’ N, 00º 20’ W). Trees were pruned to vase shape, planted at 4 m × 3 m and 4 m × 5 m spacing, depending on the orchard, on a loamy clay soils, with drip irrigation. Fertilization, pest management, thinning, and pruning were in accordance with normal commercial practices.

The experiment consisted of collecting apical meristems of the current shoots in different dates from June to September, coinciding with floral bud inductive period and floral bud differentiation period. Shoots were labeled and floral bud differentiation evaluated from the new sprouted shoots.

In the first year of the experiment (2014), twenty ‘Algerie’ loquat trees grown in Callosa were used. From late June to mid-September, 15 apexes in total were sampled at 15 days intervals.

In the second year (2015) ten ‘Algerie’ (Valencia and Palermo) and three ‘San Filippara’ (Palermo) loquat trees were used in the experiment. From early June to mid-August, 15 apexes in total from the current shoots were sampled every 5 days (approximately). In ‘Algerie’ Palermo trees, an additional 35 apexes from premature shoots were sampled in two key dates, 1$^{st}$ and 31$^{st}$ of July. Apexes from premature shoots sprouted because the removal of current shoots apexes on the 1$^{st}$ of July, were sampled from 23$^{th}$ July to 8$^{th}$ of September. Those from shoots sprouted because the removal of current shoots apex on 31$^{st}$ of July, were sampled from 24$^{th}$ August to 2$^{nd}$ October.

In the third year of the experiment fifteen ‘Algerie’ loquat trees (Valencia) were used. From mid-July to early September, 8 apexes in total from the current shoots were collected at 5-days intervals.

Collected meristems were immediately frozen with liquid $N_2$ and stored at -80°C until analyzed. Half of them were used for RNA extractions and RT-PCR analysis to analyze *FLOWERING LOCUS T (FT), TERMINAL FLOWER 1 (TFL1), LEAFY (LFY)* and *APETALA 1 (AP1)* expression; other half were used for GAs, IAA, CKs and ABA contents analysis by UHPLC/Mass spectrometer.
In all experiment, floral bud differentiation was evaluated late in October.

### 1.2. Section 2. Determination of the effect of summer temperature in floral bud differentiation

Experiments were conducted in 2014-2015 on six-years-old 'Algerie' loquat trees *Eriobotrya japonica* (Lind.) grafted onto seedling rootstock, using different trees each year, and grown at the experimental facilities, fields and greenhouses, of the Universitat Politècnica de València, Spain (39º 29' N, 00º 20' W). Trees were similar in size, vigour and potential crop load. Trees were grown in 20L-pots, under the same conditions of soil and irrigation. Fertilization, pest management, thinning, and pruning were in accordance with normal commercial practices.

In 2014, twenty trees were grown under field conditions until the $24^{th}$ of June, whereas fourteen trees were shifted to a greenhouse until late October, when the trees under field conditions were in bloom. In 2015, trees were separated into six groups of trees (A, B, C, D, E, and F) of three trees each. On the $24^{th}$ June three groups (A, B and C) were shifted to the greenhouse; on the $15^{th}$, group A was moved outdoors and group D shifted indoors, and on the $27^{th}$ July groups B and E were moved outdoors and indoors, respectively (Figure S1). Group C remained indoors through the experiment, while group F remained in the field as a control. Groups C, D and E remained indoors up to late in October, when the control trees were in bloom

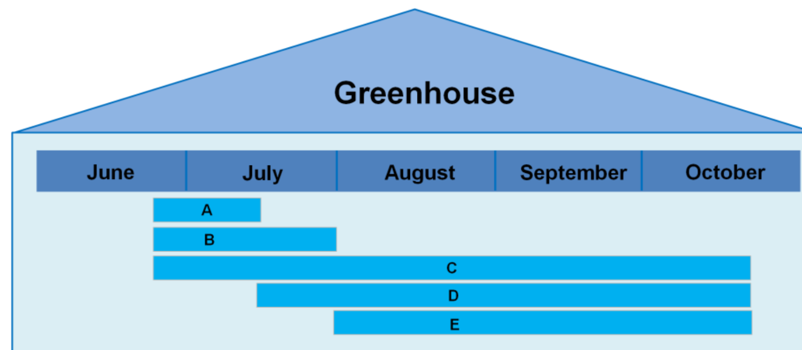


**Figure S1**. Periods under indoors conditions for each group of trees.

In both years, greenhouse temperature was set to no exceed 25 °C. Consequently, whereas during the summer maximum temperature in the field ranged 30-40 °C and minimum 20-25 °C, in the greenhouse they ranged 24-26 °C and 18°C-21°C, respectively (Figures S2 and S3).

The average relative humidity was 66% in the field and 80% in the greenhouse. The photoperiod in the greenhouse was adjusted to that of the field, it being, along the experiment, from 6:00 am to 22:00 pm, approximately; radiation did not exceed 150 watts per square meter.

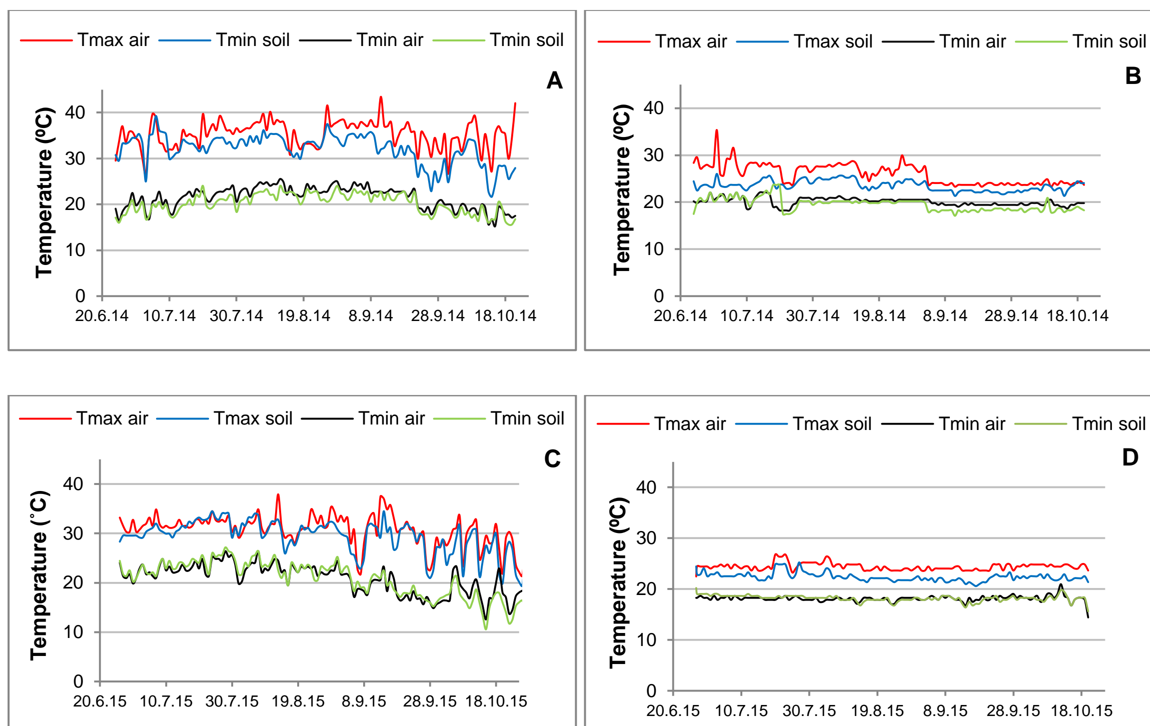


**Figure S2.** Maximum and minimum temperature in the field (A and C) and indoors (B and D) from June to October. Values for 2014 (A and B) and in 2015 (C and D).

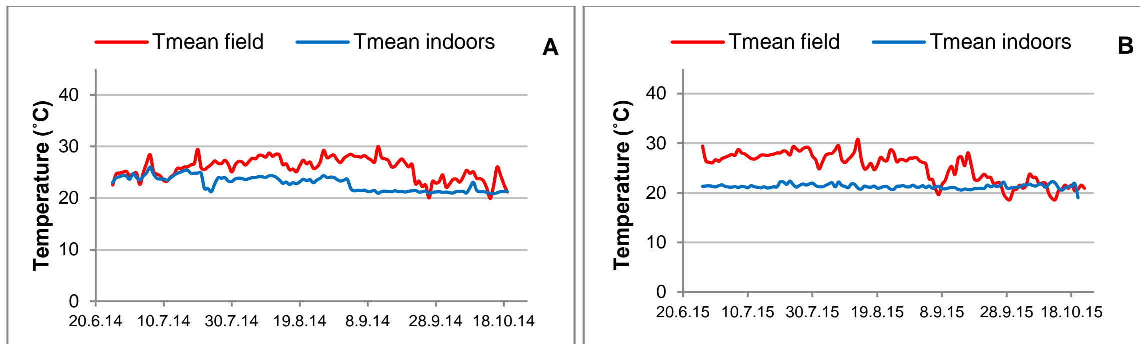


**Figure S3.** Average temperature in the field (A) and indoors (B) from June to October. Values for 2014 (A) and in 2015 (B).

Late in October, floral bud differentiation was evaluated in current and premature shoots.

Apical meristems were sampled from greenhouse and field trees from late June to late October. Three samples per date were collected and immediately frozen in liquid $N_2$ and stored at -80°C until analyzed. Half of them were used for RNA extractions and RT-PCR analysis; *FLOWERING LOCUS T (FT), TERMINAL FLOWER 1 (TFL1), LEAFY (LFY) AND APETALA 1 (AP1)* expression was determined; the other half was used for gibberellins (GAs), indole-3-acetic acid (IAA), cytokinins (CKs) and abscisic acid (ABA) analysis.

## Phenological and climatic data

Phenological observations were collected from 'Algerie' loquat trees located at Callosa, Alicante, Spain (38°39′N; 00°07′W) and Palermo, Sicily, Italy (38°04′N; 13°25′E), during 2004 - 2015 (see Appendix 1). Weather stations close to the experimental fields provided daily meteorological data.

## Functions and parameters

Two temperature response functions were in the phenology model:

### Wang

This function was first defined by Wang and Engel (1998). It has an optimum and is not symmetric. It pertains to the family of the beta functions. It has three parameters *Topt*, *Tmin* and *Tmax*.

$f_{Wang}$= Max [(2 $(T_d - T_{min})^{\alpha} (T_{opt} - T_{min})^{\alpha} - \frac{(Td - Tmin^{2\alpha}}{(Topt - Tmin^{2\alpha}}$), 0]

with $\alpha = \ln(2)/\ln(\frac{Tmax - Tmin}{Topt - Tmin})$.

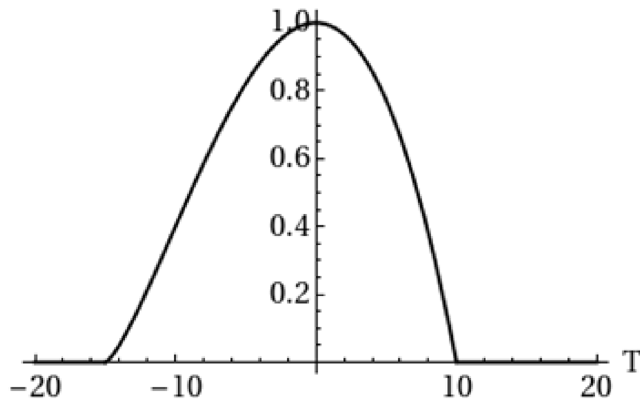


**Figure S4.** Function *fWang* with *Topt* =0.1, *Tmin* =-15, *Tmax*=10.

Richardson

This function was first introduced by Richardson *et al*. (1974), and it is a modified version of the GDD function with a plateau above the threshold parameter *Thigh*:

$f_{Richardson}$(Td)= Max(Min $(T_d - T_{low}, T_{high} - T_{low})$,0)

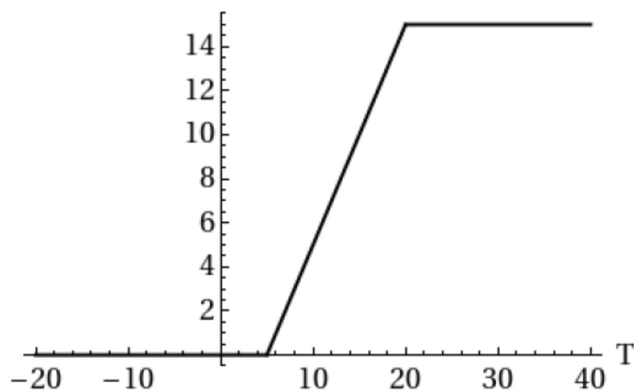


**Figure S5.** Function *fRichardson* with *Thigh*=20, *Tlow*=5.

The best model was selected based on two criteria: the model with the highest efficiency, i.e. that gives the highest percentage of variance explained (EF); Greenwood *et al.*, 1985; Eqn 1) where a negative value indicated that the model performed worse than the null model (mean date of flowering), and a value above zero indicated that the model explained more variance than the null model (with a maximum value of 1); and the root means squared error (RMSE; Eqn 2), which gives the mean error of the prediction in days where $O_i$ is the observed value, $S_i$ is the simulated value, $\bar{O}$ is the mean observed value of the dataset used, n is the number of observations, k is the number of parameters.

$$EF = 1 - \left( \frac{\sum_{i=1}^{n} (S_i - O_i)^2}{\sum_{i=1}^{n} (O_i - \bar{O})^2} \right) \quad \text{(Eqn 1)}$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n} (S_i - O_i)^2}{n}} \quad \text{(Eqn 2)}$$

### 1.3. Section 3. Determination of floral bud differentiation by means of molecular and microscopic analysis.

Twenty-years-old 'Algerie' loquat trees grafted onto seedling rootstock were used in this experiment. Trees were grown under the same conditions of soil and irrigation, in the experimental field and greenhouse at the Universitat Politècnica de València, Spain (39º 29' N, 00º 20' W). Trees were pruned to vase shape, planted 4 m × 3 m apart on a loamy clay soil, with drip irrigation. Fertilization, pest management, thinning, and pruning were in accordance with normal commercial practices.

From mid-July to mid-September, 8 apexes were sampled at 5 days intervals, immediately frozen with liquid $N_2$, and stored at -80°C until analyzed. Half of them were used for RNA extractions and RT-PCR analysis for the study of *TERMINAL FLOWER 1 (TFL1), LEAFY (LFY)* and *APETALA 1 (AP1)* expression, and analysis of ABA content by using UHPLC/Mass spectrometer.

The other half was fixed under vacuum with karnofsky solution for microscopic studies.

## 2. Methods

### 2.1. Gene expression analysis by qRTPCR

Total RNA was extracted from frozen tissues and subsequently treated with DNase I (RNase Free DNase Set, Qiagen, USA). The amount of RNA was measured by spectrophotometric analysis (NanoDrop NDB1000 spectrophotometer, Thermo Fisher, USA). The absence of DNA contamination was checked by performing a no reverse transcription assay which consisted of a PCR with each RNA sample using the Loquat actin primers (Table S1). No amplified products were detected, which confirmed the purity of the RNA extracts.

The transcripts present in 1 µg of total RNA were reverse-transcribed using the QuantiTect® Reverse Transcription Kit (Qiagen, USA) in a total volume of 20 µl. A 2.5 µl aliquot of a 4-time diluted first-strand cDNA was used for each amplification reaction. Quantitative real-time PCR was carried out on a Rotor Gene Q 5-Plex (Qiagen, USA) using the QuantiTect® SYBR® Green PCR Kit (Qiagen, USA). The reaction mix and conditions followed the manufacturer's instructions with certain modifications. The PCR mix contained 2.5 µl of diluted cDNA, 12.5 µl of QuantiTect® SYBR Green PCR Master Mix (Qiagen, USA), 1.5 µl of 0.3 µM primer F, and 1.5 µl of 0.3 µM primer R, the final volume being 25 µl. The cycling protocol for the amplification consisted of 15 min at 95ºC for pre-incubation, then 40 cycles of 15 s at 94ºC for denaturation, 30 s at 60ºC for annealing and 30 s at 72ºC for extension. RT-PCR reactions were repeated three times for each gene and monitored in real time with the Rotor Gene Detector. After amplification, the melting-curve analysis excluded artefactual amplifications. The relative expression of RNA transcripts was quantified with the threshold cycle values (Ct) obtained from each sample using the 2-DDCt method (Livak and Schmittgen, 2001). Expression levels were calculated relative to the constitutively expressed ACTIN gene (Table S1). The relative gene expression level is given by 2-DDCt. Normalization was performed to the lowest value between the samples or to the first sampled date. Two or three

independent biological samples under each experimental condition were evaluated in technical triplicates.

**Table S1**. Primer sequence used in RT-PCR amplification reactions.

| Annotation | NCBI accession number | 5′ -Direct primer- 3′<br>5’-Reverse primer- 3’ | Predicted product (bp) |
|---|---|---|---|
| *ACTIN* | JX089586 | ATGAGGGAGGGCATAACC<br>TGTTGCCATACAGGCTGT | **121** |
| *EjLFY* | AB162033 | ATCCAGGTCCAGAACATTGC<br>ATGTAGCTTGCGCCTGACTT | **100** |
| *EjAP1* | AY880262 | AGCTGGACCTGACTCTGGAA<br>TGATGATCAAGCAGCAAAGC | **65** |
| *EjTFL1* | GU320722 | TCTGTTGTCACAGCCAAACC<br>AGTGCAGGTGCTCCCTTAGA | **65** |
| *EjFT1* | KP941774 | TCTCAGGGTGACCTACGGTAC<br>ATCGTCGCCACCAATATCAG | **99** |
| *EjFT2* | KP941772 | AGCTGGTGGGGGATACTATACG<br>GTGCGCACACATCGATAGAC | **114** |

### 2.2. Hormone isolation, purification and quantification

Material frozen in liquid nitrogen was ground into a fine powder. Aliquots (about 50 mg dry weight) of material were extracted with 80% methanol containing 1% acetic acid. Internal standards were added and mixed with the aliquots at 4°C for 1 hour. The internal standards for quantification of each of the different plant hormones were the deuterium-labelled hormones. The extraction protocol used is that described in Seo *et al.* (2011) with certain modifications. In brief, for desalination, the extracts were passed through reverse phase columns HLB (Waters). The plant hormones were eluted with 80% methanol containing 1% acetic acid and consecutively applied to cation exchange MCX columns (Waters). The fraction containing the acidic ABA, GAs, IAA, isopentenyl adenine (iP), trans-zeatina (*t*Z) and dihydrozeatin (DHZ) was applied through ion exchange WAX columns (Waters). The final residue was dissolved in 5% acetonitrile-1% acetic acid, and the hormones were separated using an auto sampler and reverse phase UPHL chromatography (2.6 µm Accucore RPMS column, 50 mm length x 2.1 mm i.d.; ThermoFisher Scientific) with a 5 to 50%

acetonitrile gradient containing 0.05% acetic acid, at 400 µL/min for 14 min. The hormones were analyzed with a Q-Exactive mass spectrometer (Orbitrap detector; ThermoFisher Scientific) 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. Samples were analysed by triplicate.

### 2.3. Model parameterization

Models were parameterized using the Phenology Modelling Platform software (http://www.cefe.cnrs.fr/fr/recherche/ef/forecast/phenology-modelling-platform) (Chuine *et al.*, 2013). This software proposes fitting different types of phenological models, either selected in a library or defined by the user, using a simulated annealing algorithm following Chuine *et al.* (1998). The optimization algorithm minimizes the mean-squared error, that is the squared difference between the observed dates and the dates predicted by the model.

### 2.4. Microscopic analysis

Sampled of apical bud were fixed in karnofsky solution (Glutaraldehyde-paraformaldehyde) and conserved at -4 until the next step. Tissue was embedded in LR-White resin. LR-White embedding process includes 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 in an oven with temperature set at 60°C.

Embedded material was sectioned at 2 microns 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 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

Analysis of variance was performed on the data, using the least significant difference (LSD) test for means separation. Standard errors of means were calculated and reported in the figures and tables to indicate variations among samples. The experimental data were analyzed with Statgraphics Plus 5.1 software (Statistical Graphics, Englewood Cliffs, NJ).

# ***Results***

# 1. Section 1. The influence of the time of shoot apex removal on the floral bud differentiation of premature shoots

In loquat, apex of a current shoot changes from vegetative to reproductive stage during summer, i.e. under high temperature conditions. Indeed, just before floral bud differentiation, a decline in the growth rate due to high temperature takes place. To establish the role of this 'summer rest period' on the apex transition from vegetative to reproductive stage, sprouting was promoted at different times, before, during and after floral bud differentiation occurred.

In this section, the effect of the time of current shoot apex removal was studied for two cultivars (Algerie and San Filippara) in two growing areas (Palermo and Valencia).

**Figure 1.1.** The influence of the time of current shoot apex removal on the percentage of reproductive secondary shoots in loquat. Evaluation was carried out on the 31st of October. Values are the mean of 15 meristems from 'Algerie' and 'San Filippara' loquat trees.

Current shoot apex removal promotes new shoots (secondary shoots) to sprout from the buds nearest to the removed apex, the date of removal determines floral bud differentiation. In our experiment, the percentage of the new reproductive shoots was inversely and significantly related to the date of apex removal (r = -0.607; $P \leq 0.01$) (Figure 1.1). Thus, when the current shoot apex was removed from early to mid-July, i.e. between floral bud induction and the transition to reproductive stage, most of the new sprouts (40% to 50% on average) developed into panicle. Afterwards, from mid-July to mid-August, the

percentage of the new shoots developing into panicle continuously decreased to became zero from 20 August onwards (Figure 1.1), developing vegetative.

This trend was similar regardless of the year, the growing area, and the cultivar (Figure 1.2 A, B and C, respectively). Nevertheless, for the same cultivar (Algerie) a quantitative difference between years was observed. In the experiment carried out in 2015, date of apex removal and percentage of flowering shoots relationship was significantly (r = -0.711; $P \leq 0.05$) but lesser than that for 2014 (r = -0.988; $P \leq 0.01$) and for 2016 (r = -0.901; $P \leq 0.01$) (Figure 1.2 A). In 2015 the first date of apex removal was hastened to mid-June, coinciding with the floral bud inductive period. Removing apex shoot in a date so distant from the floral bud differentiation gave rise a higher percentage of vegetative shoots. The possibility of an interference with the floral bud induction caused by the 2015 environmental conditions cannot be discarded.

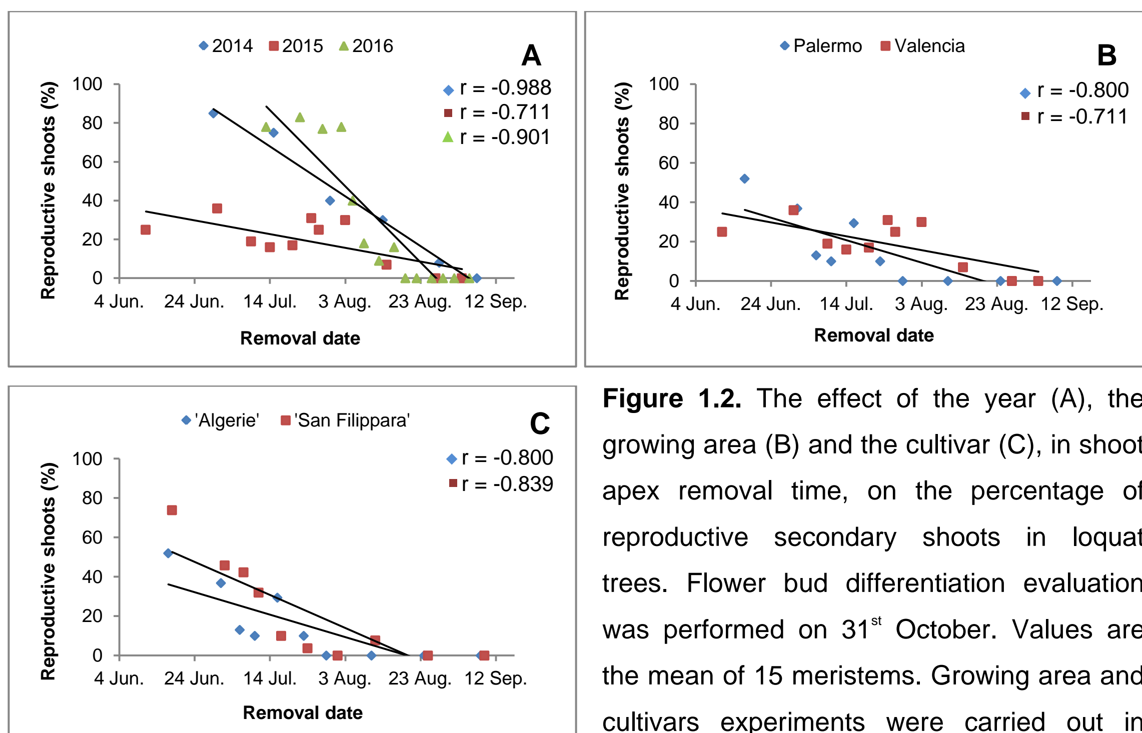


**Figure 1.2.** The effect of the year (A), the growing area (B) and the cultivar (C), in shoot apex removal time, on the percentage of reproductive secondary shoots in loquat trees. Flower bud differentiation evaluation was performed on 31st October. Values are the mean of 15 meristems. Growing area and cultivars experiments were carried out in 2015

Notwithstanding, for 2015 the date of apex removal and percentage of new flowering shoots relationships for the same cultivar (Algerie) also displayed inversely, and very similar for the two locations, Palermo (r = -0.800; $P \leq 0.01$); and Valencia (r = -0.711; $P \leq 0.05$) (Figure 1.2 B). Furthermore, for the same year

(2015) and location (Palermo), regression analysis for the two cultivars, Algerie and San Filippara, were very similar, r = -0.800 ($P \leq 0.01$) and r = - 0.839 ($P \leq 0.01$), respectively (Figure 1.2 C).

Accordingly, in loquat there is an inverse relationship between the date of apex removal and percentage of new flowering shoots irrespective of the year, location and cultivar, suggesting that the earlier a bud sprouts the higher the probability to develop into a panicle.

### 1.1. Flowering genes expression in apices from current shoots and secondary shoots arose in July and August

In Palermo, using 'Algerie' loquat trees, the apex removed from current shoots and growing apex from secondary shoots were sampled. When removing the apex on the 1st of July, secondary shoots emerged around 20 days later, coinciding when the current-year shoot was preparing the transition from vegetative to reproductive stage and when the external and internal conditions were favorable for floral bud differentiation. However, growing apexes of secondary shoots sprouted after removing that from the current-shoot on the 31st July, emerged in late August, when the most of current shoots have already undergone floral differentiation process and environmental conditions were changing. Genetic analysis was performed in order to see differences in the flowering genes expression between apexes from current shoots and from secondary shoots emerged in July and August.

In the main apex, the expression of *EjLFY* and *EjAP1* (Figure 1.3 and 1.4), displayed parallel mRNA transcript stayed at low levels until mid-August., increasing afterwards and reaching maximum values in early September, coinciding with the floral bud differentiation period. The secondary shoots, sprouted after removing the main apex on 1st July, were sampled from 23th July to 8th September. Time course of *EjLFY* and *EjAP1* expression was also similar. mRNA transcript accumulation remained constant and at low level for the studied period. Only on 8th September a slightly increase was appreciated in both genes.

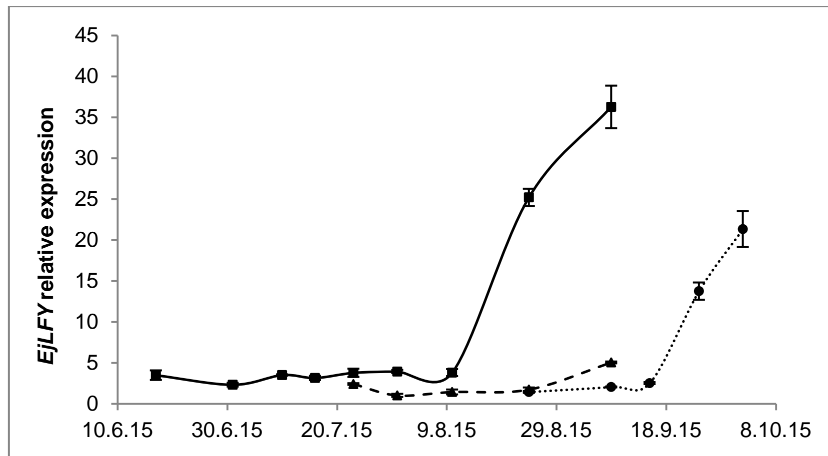


**Figure 1.3.** *EjLFY* relative expression pattern of 'Algerie' loquat apex of current shoots (solid line), and secondary shoots sprouted after removing the main apex on 1st July (broken line) and 31st July (dotted line). Data are means ± ES of 3 qRT-PCR replicates. In some case SE is smaller than the symbol size. Normalization was performed to the lowest value between the samples

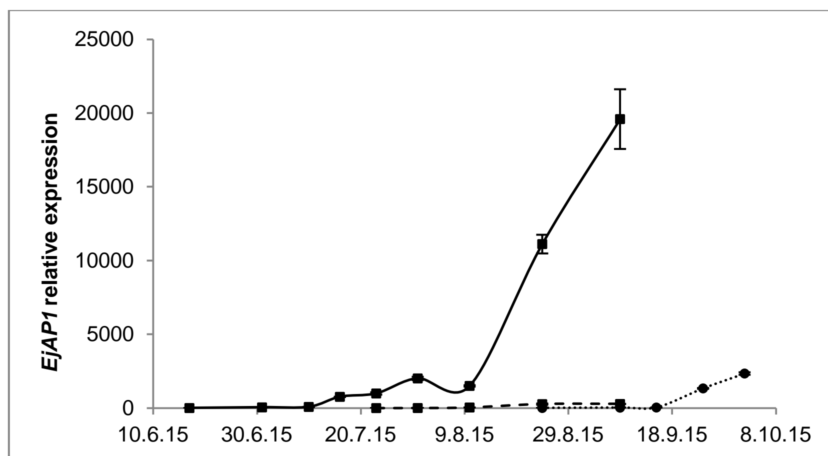


**Figure 1.4.** *EjAP1* relative expression pattern of 'Algerie' loquat apex of current shoots (solid line), and secondary shoots sprouted after removing the main apex on 1st July (broken line) and 31st July (dotted line). Data are means ± ES of 3 qRT-PCR replicates. In some case SE is smaller than the symbol size. Normalization was performed to the lowest value between the samples.

The secondary shoots, sprouted after removing the main apex on 31st July, were sampled from 24th August to 2th October. Low level of expression was found for both genes, *EjLFY* and *EjAP1*, until mid-September. However, in late September *EjLFY* expression increased reaching levels five-fold higher than that of 31st July removed apex, whereas *EjAP1* expression, which also increased during the

same dates, reached a very much lower relative expression than that of the main apex.

The flowering repressor *EjTFL1* (Figure 1.5) expressed higher in mid-June in the apices from current shoots. Afterward, expression decreased gradually until late July, before the start of floral bud differentiation. The secondary shoots sprouted after removing the apex on 1st July and on 31st July displayed similar trend; the expression was higher during the first dates and then decrease. Secondary shoots sprouted in August displayed a higher expression of *EjTLF1* during the floral bud differentiation, and could be related to the unfitness of these buds for differentiate a panicle.

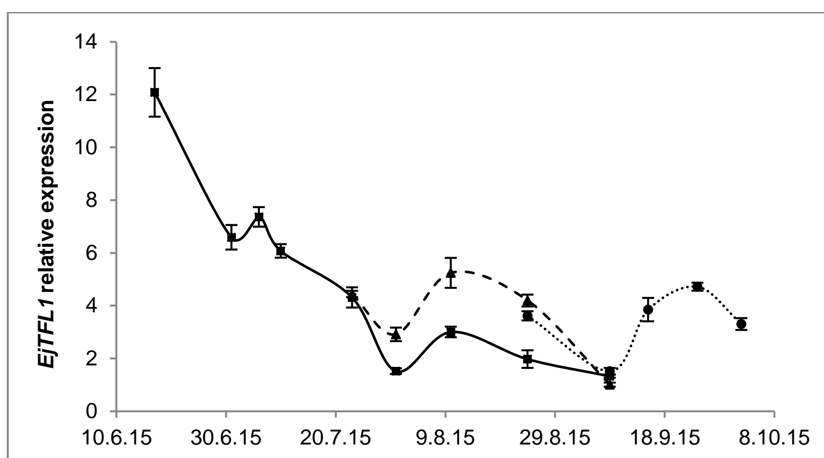


**Figure 1.5.** *EjTFL1* relative expression pattern of 'Algerie' loquat apex of current- shoots (solid line), and secondary shoots sprouted after removing the main apex on 1st July (broken line) and 31st July (dotted line). Data are means ± ES of 3 qRT-PCR replicates. In some case SE is smaller than the symbol size. Normalization was performed to the lowest value between the samples.

The expression of *EjFT1* (Figure 1.6) in the main apex showed a fluctuating pattern resulting in different expression peaks during the period studied. Secondary shoots sprouted in July had a similar behaviour. Secondary shoots sprouted in August also presented a similar expression and time course, although delayed in time.

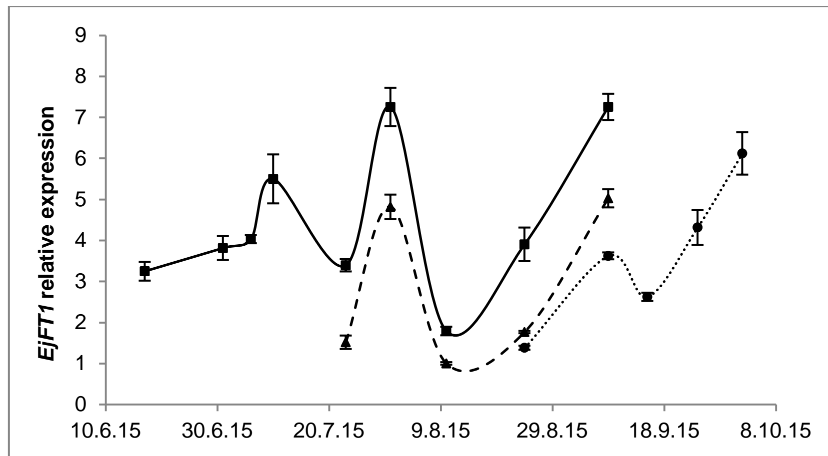


**Figure 1.6.** *EjFT1* relative expression pattern of 'Algerie' loquat apex of current shoots (solid line), and secondary shoots sprouted after removing the main apex on 1st July (broken line) and 31st July (dotted line). Data are means ± ES of 3 qRT-PCR replicates. In some case SE is smaller than the symbol size. Normalization was performed to the lowest value between the samples.

### 1.2. Hormonal content in apexes from current shoots and from secondary shoots arose in July and August

ABA content in the apex of current shoots showed maximum concentration in mid-June (Figure 1.7). Afterward, it fell and remained low until late August when increased slightly. In the secondary buds arose in July ABA content, increased moderately over time and dropped in mid-July. Its concentration was always lower than that of the apex of current shoots. Secondary buds arisen in August displayed a lower concentration than, current and secondary shoots of July during the first sampling dates, decreasing afterwards and reaching a peak in late September.

In current shoots $GA_1$ also displayed a maximum concentration in mid-June (Figure 1.8). Afterward, fell to near zero during the period of the experiment. In the secondary shoots emerged in July, the concentration remained fairly constant and higher than that of current shoots for the same period. In the secondary shoots emerged in August, $GA_1$ concentration was similar to that of the latter but peaking on 24th September.

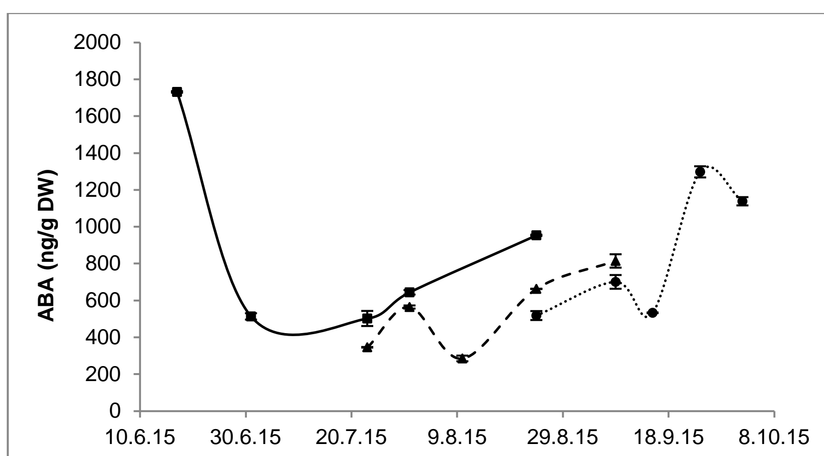


**Figure 1.7.** Time-course of ABA concentration in ‘Algerie’ loquat apex of current shoots (solid line), and secondary shoots sprouted after removing the main apex on 1st July (broken line) and 31st July (dotted line). Data are means ± ES of three replicates. In some case SE is smaller than the symbol size.

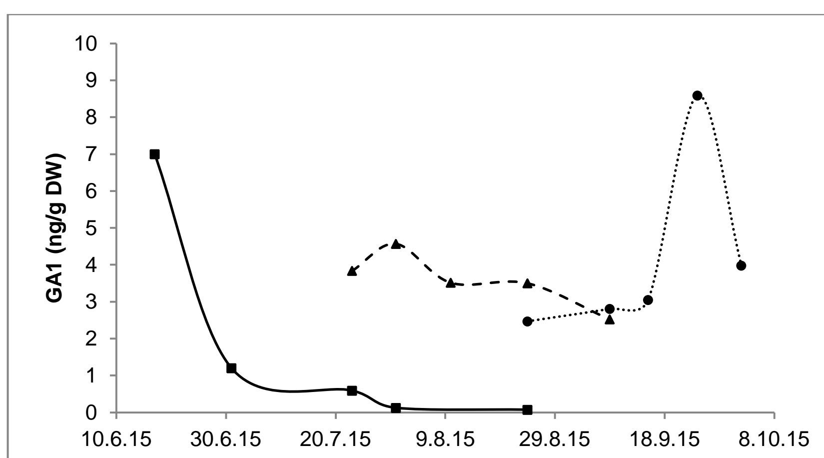


**Figure 1.8.** Time-course of $GA_1$ concentration in ‘Algerie’ loquat apex of current shoots (solid line), and secondary shoots sprouted after removing the main apex on 1st July (broken line) 31st July (dotted line). Data are means ± ES of three replicates. In some case SE is smaller than the symbol size.

IAA concentration in current shoots showed the highest concentration in mid-June (Figure 1.9). Then, the concentration fell and remained constant up to the end of the experiment. In the secondary shoot emerged on July and on August, concentration remained almost constant up to the end of the experiment with values between 15 and 30 ng/g dry weight.

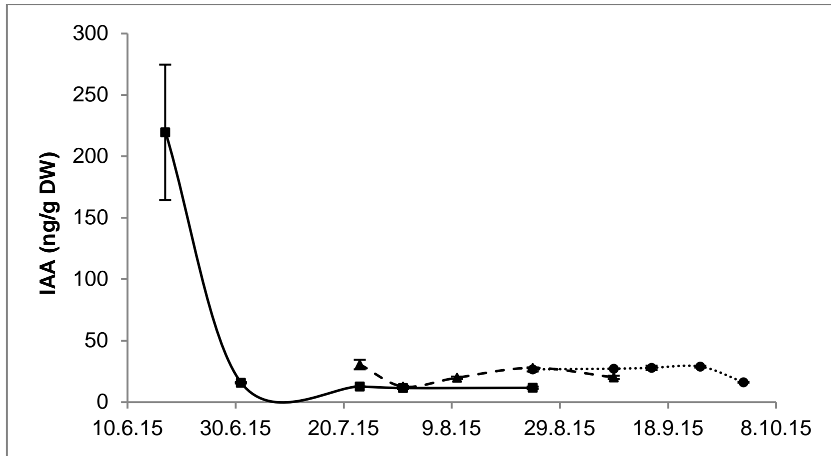


**Figure 1.9.** Time-course of IAA concentration in 'Algerie' loquat apex of current shoots (solid line), and secondary shoots sprouted after removing the main apex on 1st July (broken line) and 31st July (dotted line). Data are means ± ES of three replicates. In some case SE is smaller than the symbol size.

*tZ* concentration decreased in current shoots and secondary shoots of July (Figure 1.10); the latter displaying the higher *tZ* concentrations. Secondary shoots emerged in August showed very low tZ concentrations.

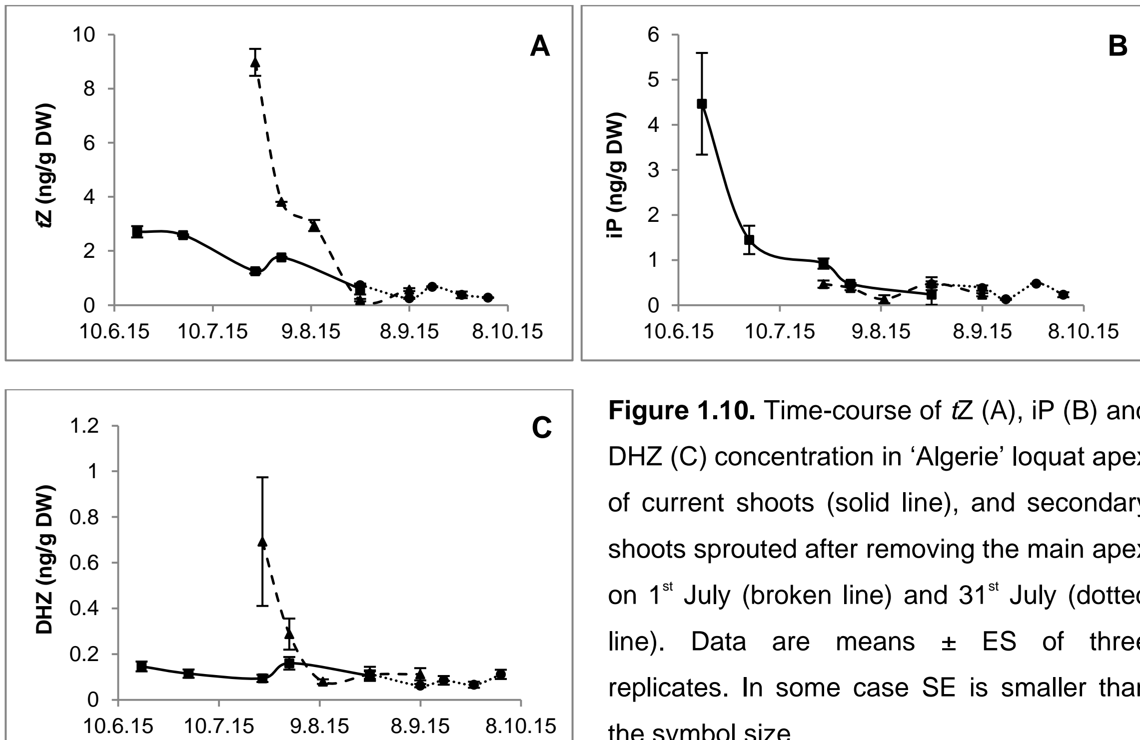


**Figure 1.10.** Time-course of *tZ* (A), iP (B) and DHZ (C) concentration in 'Algerie' loquat apex of current shoots (solid line), and secondary shoots sprouted after removing the main apex on 1st July (broken line) and 31st July (dotted line). Data are means ± ES of three replicates. In some case SE is smaller than the symbol size.

IP presented a maximum in the current shoots in mid-June, falling afterwards and remained in a low level up to the end of the experiment. IP concentration in both secondary shoots emerged in July and in August presented fairly low

values for the whole period studied. Finally, DHZ concentrations of current and secondary shoots were very low. The highest concentration was found in the secondary shoots emerged in July during the first sampling dates.

## 2. Section 2. The effect of summer temperature on floral bud differentiation

A significant relationship between flowering and air temperature during summer, i.e. during floral bud differentiation period was observed. Trees grown indoors (average maximum air temperature around 25 ˚C) from late June to late October were unable to flower in contrast to those growth in field conditions (see Figure S2) (Table 2.1).

**Table 2.1.** The influence of temperature during floral bud differentiation period, on the percentage of vegetative and reproductive shoots in 'Algerie' loquat trees at flowering date (505 BBCH). Trees grew indoors (Tmax ≈ 25ºC) from 24th June (10 days after floral bud induction, approximately) to 20th October. Values are the mean of 14 trees for greenhouse and 6 trees for field.

| | Vegetative shoots (%) | Floral shoots (%) |
|---|---|---|
| **Field** | 3 | 97 |
| **Indoors** | 100 | 0 |

Similar results were observed the second year experiment. In this case, floral bud differentiation was also strongly suppressed in all groups of trees kept in greenhouse regardless of the time they were below an average maximum temperature of 25˚C, approximately [from 118 days (C), the highest, to 20 days (A), the lowest]. In contrast, all trees grown under field conditions flowered as usual (95% of theirs buds differentiated into a panicle) (Table 2.2). It is important to note that only 13% of the buds of the trees remaining indoors the shortest period (group A) developed into flowers, in contrast to the control trees grown in the field (group F) that did it 100%. Just 20 days under greenhouse conditions in early summer were enough to suppress the floral bud differentiation process. In the groups (B, C, D and E) no floral bud differentiation signals were detected at all, whereas all buds of the trees of the group F flowered, as the shoot continued emerging new leaves.

No differences between groups of trees growing indoors were detected irrespective of the period remaining indoors (Table 2.2).

**Table 2.2.** The influence of temperature during floral bud differentiation period, on the percentage of vegetative and reproductive shoots in 'Algerie' loquat trees at flowering date (505 BBCH). Trees grew indoors (Tmax ≈ 25ºC) from 24th June (10 days after floral bud induction approximately) to 20th October. Values are the mean of 3 trees for group.

| Groups | Indoors period | Vegetative shoots (%) | Floral shoots (%) |
|---|---|---|---|
| **A** | 24 June- 15 July | 87 | 13 |
| **B** | 24 June- 27 July | 100 | 0 |
| **C** | 24 June- 20 October | 100 | 0 |
| **D** | 15 July- 20 October | 100 | 0 |
| **E** | 27 July- 20 October | 100 | 0 |
| **F** | Outdoors | 5 | 95 |

## 2.1. Flowering gene expression and hormonal content under different temperature conditions during floral bud differentiation

Significant differences in the seasonal level of mRNA expression of *EjLFY* in the meristem were detected between trees under field and greenhouse conditions. Whereas the maximum *EjLFY* expression was observed in the 11th August in the former (22-fold relative to initial levels), the *EjLFY* expression in the latter remained lower and almost constant during the period of the study (Figure 2.1).
Twenty five days later, accumulation of *EjLFY* transcript in buds of field trees were reduced to 0.56-fold, coinciding with the first visible signal of floral bud differentiation (501 BBCH).

Similar trend was observed for *EjAP1* expression, the maximum mRNA accumulation for trees under field conditions being also on the 11th August (Figure 2.2); its accumulation transcripts, however, was greater (up to 4700) than that for *EjLFY* (up to 25) (Figure 2.1 and 2.2). Since relative expression of *EjLFY* and *EjAP1* transcript in buds of trees grown under field conditions was significantly higher than that of buds of trees grown indoors and no flowers were developed on the latter, floral bud differentiation should begin in mid-August, when the temperature stimulus remains higher in the field.

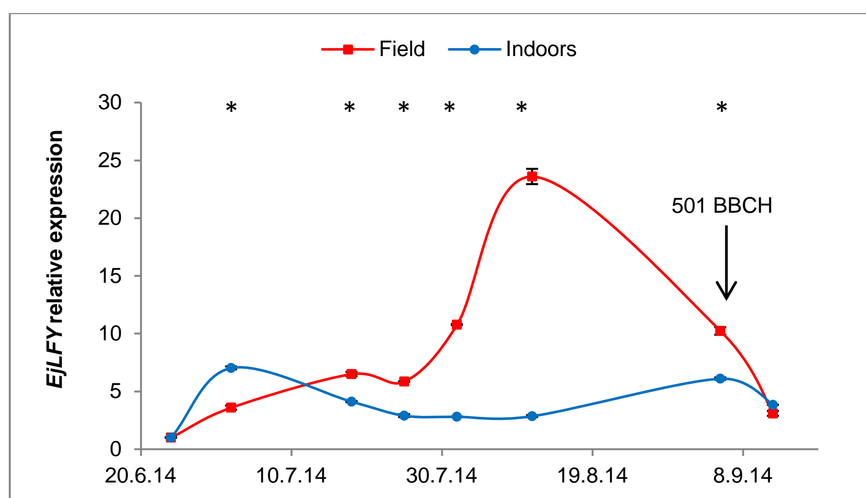


**Figure 2.1.** Relative expression pattern of *EjLFY* in apex shoots of 'Algerie' loquat trees grown under field (Tmax between 25-40ºC) and indoors conditions (Tmax ≈ 25ºC). Data are means ± ES of 3 qRT-PCR replicates. Normalization was performed to the first sample date. * indicate significant differences (P ≤ 0.05). In some case SE is smaller than the symbol size. Values for 2014.

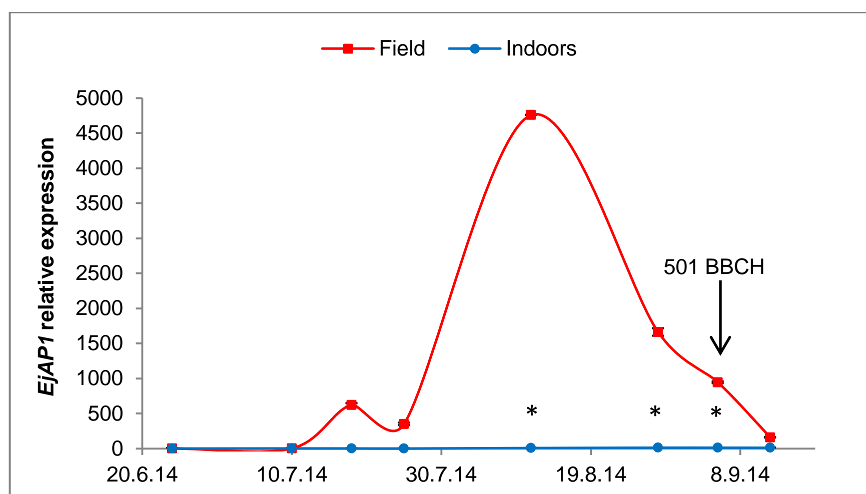


**Figure 2.2.** Relative expression pattern of *EjAP1* in apex shoots of 'Algerie' loquat trees grown in field conditions (Tmax between 25-40ºC) and under indoors conditions (Tmax ≈ 25ºC). Data are means ± ES of 3 qRT-PCR replicates. Normalization was performed to the first sample date. * indicate significant differences (P ≤ 0.05). In some case SE is smaller than the symbol size. Values for 2014.

No differences in *EjTFL1* expression trend in buds were detected between trees growing under field and indoors conditions, except at early September when the latter reached a second peak of *EjTFL1* expression (Figure 2.3). However, 8 days after the transfer of the trees to indoors, levels of *EjTFL1* transcript accumulation increased 1.6-fold relative to the levels in trees growing under field conditions, and remained significantly higher during whole July, falling dramatically 20 days later (Figure 2.3).

It is important to note that the minimum *EjTFL1* expression observed in buds of trees growing under field conditions from late July onwards coincided with the maximum *EjLFY* and *EjAP1* expression (Figure 2.1, 2.2 and 2.3). These result suggest that the accumulation of floral meristem identity genes transcript (*EjLFY* and *EjAP1*) is allowed by the low expression of the floral gene repressor *EjTFL1.*

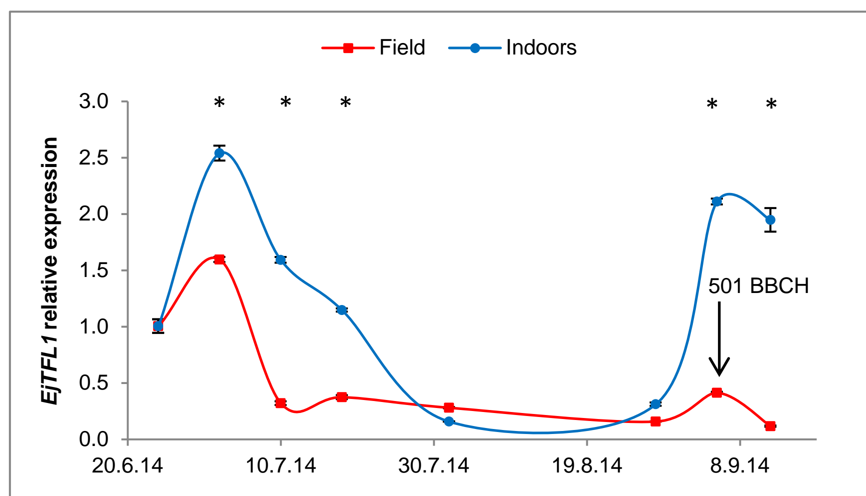


**Figure 2.3.** Relative expression pattern of *EjTFL1* in apex shoots of ‘Algerie’ loquat trees grown under field (Tmax between 25-40ºC) and indoors conditions (Tmax ≈ 25ºC). Data are means ± ES of 3 qRT-PCR replicates. Normalization was performed to the first sample date. * indicate significant differences ($P \leq 0.05$). In some case SE is smaller than the symbol size. Values for 2014.

Nevertheless, this relationship between floral meristem identity promoters and repressors genes was not detected in trees growing indoors indicating that *EjLFY* and *EjAP1* expression was unaffected by *EjTFL1* expression (Figure 2.1, 2.2 and 2.3).

Significant differences in the two loquat *FLOWERING LOCUS T (FT)*-like genes, *EjFT1* and *EjFT2*, behaviour were also observed (Figure 2.4). Whereas no *EjFT2* transcript was detected in buds of both, field and indoors trees, accumulation of *EjFT1* transcript showed a different trend between them. Levels of *EjFT1* transcript accumulation in buds of trees grown in field conditions reached the maximum in mid-July decreasing significantly 15 days later up to initial levels until the rest of the period studied (Figure 2.4). In indoors trees, *EjFT1* mRNA transcript grew slower reaching a maximum level (5-fold relative to the initial level) on 25$^{th}$ July, 15 days later than in field conditions. Afterwards, level of expression declined 0.4-fold, remaining almost constant up to mid-September, when increased again and reached the maximum level (Figure 2.4).

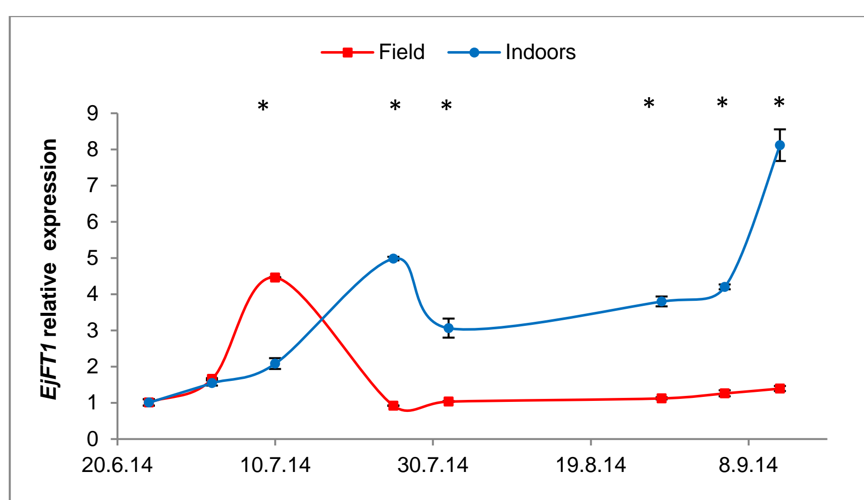


**Figure 2.4.** Relative expression pattern of *EjFT1* in apex shoots of 'Algerie' loquat trees grown under field (Tmax between 25-40ºC) and indoors conditions (Tmax ≈ 25ºC). Data are means ± ES of 3 qRT-PCR replicates. Normalization was performed to the first sample date. * indicate significant differences ($P \leq 0.05$). In some case SE is smaller than the symbol size. Values for 2014.

In spite of the reduction of the *EjFT1* relative expression reduction observed in buds of trees grown indoors at the end of July, levels of *EjFT1* transcripts remained above initials levels during the rest of the period studied, being higher than those for field trees (Figure 2.4).

It is important to note that *EjTFL1* and *EjFT1* developed similar trend in buds of trees grown in field conditions (Figure 2.3 and 2.4). Nevertheless, the role of the

latter in the flowering response, particularly in the flower bud differentiation, requires a deep study.

Temperature also modified significantly the hormone content in trees. The response depends significantly plant hormones content (Figure 2.13 and 2.14). Thus, the time-course of ABA concentration in buds of trees growing under field conditions was opposite to those growing under indoors conditions. In the former the ABA concentration decreased significantly (80%) up to the end of July and remained almost constant until the end of the period studied (1200 ng/g DW, on average), whereas in the latter increased gradually reaching the maximum at the beginning of September, becoming 14-fold higher than in field conditions (11483 ng/g DW). The response to indoors conditions was not immediate and ABA concentration was almost constant (≈ 4000 ng/g DW) during the first 15 days after the trees were transferred to indoors. Fifteen days later, significant differences were already found (4536 ng/ g DW and 786 ng/ g DW for indoors and field conditions, respectively) (Figure 2.5).

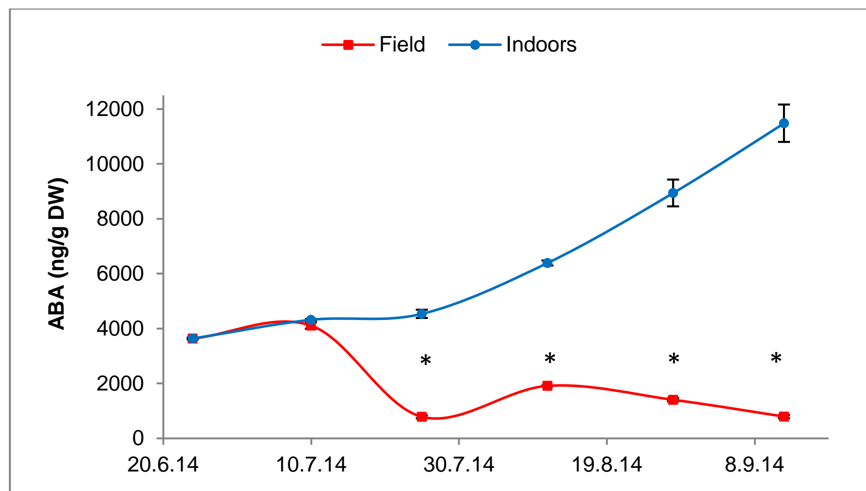


**Figure 2.5.** Time-course of ABA concentration in apex shoots of 'Algerie' loquat trees grown under field (Tmax between 25-40ºC) and indoors conditions (Tmax ≈ 25ºC). Values are means ±SE of three replicates. In some case SE is smaller than the symbol size. * indicates significant differences (P ≤ 0.05). Values for 2014.

The time course of the bioactive gibberellins concentration ($GA_1$ and $GA_4$) in buds was similar regardless of the growing conditions (Figure 2.13 and 2.14). In both cases, the concentration decreased gradually during all the period of the study. However, for $GA_1$ concentration there were not significant differences

between buds of trees grown under field and indoors conditions, whereas for $GA_4$ significant differences were found at mid-July and at the end of July (16 and 31 days after trees were transferred to the indoors conditions, respectively), (Figure 2.6 A and C). In the former, $GA_4$ concentration was higher (76%) under indoors conditions and in the latter under filed conditions (86%) (Figure 2.6 C). Nevertheless, the time-course of $GA_8$, the 2β-hydroxylated catabolite of $GA_1$, in buds of trees grown under field and indoors conditions barely showed differences between them, except for first half of August when it was more active in the former and concentration slightly increased 0,65 ng/g DW, on average (Figure 2.6 B).

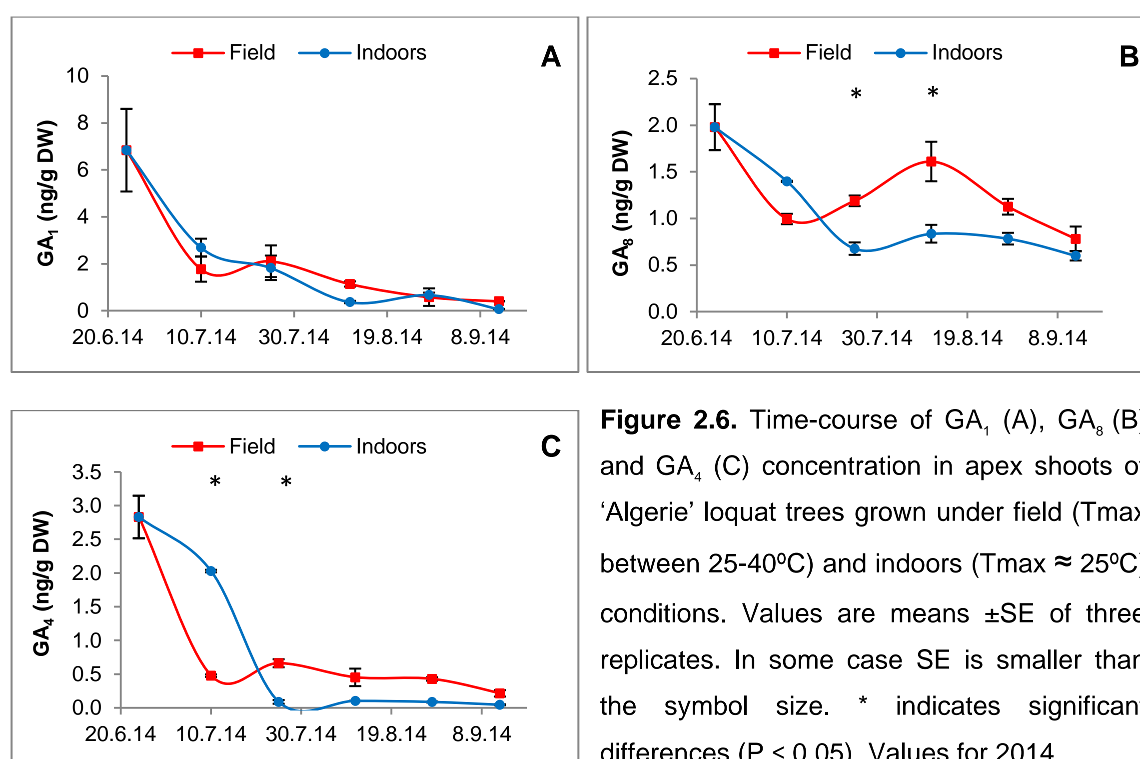


**Figure 2.6.** Time-course of $GA_1$ (A), $GA_8$ (B) and $GA_4$ (C) concentration in apex shoots of 'Algerie' loquat trees grown under field (Tmax between 25-40ºC) and indoors (Tmax ≈ 25ºC) conditions. Values are means ±SE of three replicates. In some case SE is smaller than the symbol size. * indicates significant differences ($P \leq 0.05$). Values for 2014.

It is important to note that $GA_1$ was quantitatively more relevant than $GA_4$, the maximum concentration of the former being around 2-fold higher than that of the latter (Figure 2.6 A and C).

Similar time-course for trans-zeatin (*tZ*) and indolacetic acid (IAA) concentration was observed in buds of trees growing under field and indoors conditions. In both cases, the hormone concentration decreased in time. However, under indoors conditions, *tZ* content lasted 15 days, approximately, to drop, and in late

August, when *tZ* concentration in buds of field trees continued decreasing until the end of the study, concentration in buds of the trees growing indoors increased up to 10 ng/g DW, reaching significant differences (Figure 2.7).

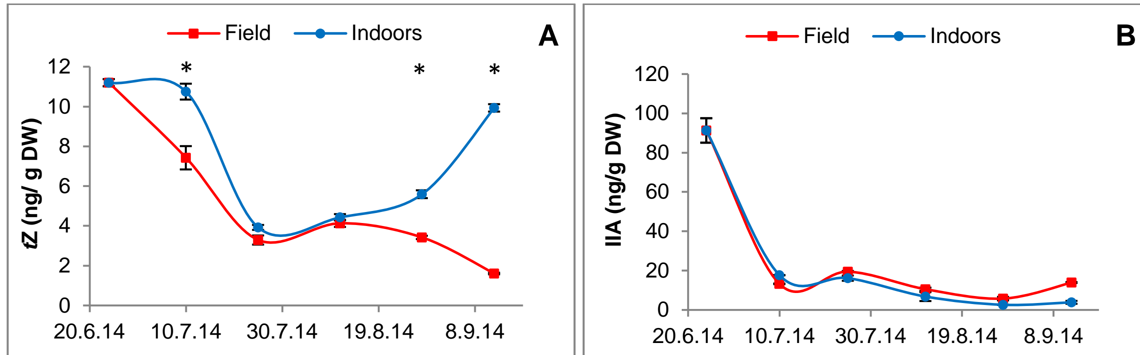


**Figure 2.7.** Time-course of *tZ* (A) and IAA (B) concentration in apex shoots of 'Algerie' loquat trees grown under field (Tmax between 25-40ºC) and indoors conditions (Tmax ≈ 25ºC). Values are means ±SE of three replicates. In some case SE is smaller than the symbol size. * indicates significant differences ($P \leq 0.05$). Values for 2014.

Not significant differences were found for IAA concentration between trees grown under field and indoors conditions. It is important to note that IAA content was markedly higher than *tZ* content, the maximum concentration being almost 10-fold higher (Figure 2.7).

In the second year of experiments, the floral meristem identity genes (*EjLFY*, *EjAP1* and *EjTFL1*) expression was also altered by means of the indoors conditions, i.e, maximum average temperature under 25ºC. *EjLFY* transcript did not show significant differences neither the date nor temperature conditions, except at the end of July when *EjLFY* transcripts in buds of the trees growing indoors was 1.3-fold higher than those growing in the field (Figure 2.8 A). However, 17 days later (mid-August) during floral bud differentiation, *EjLFY* levels were similar for both trees, in contrast to the results of the first year for which field trees displayed a marked rise in *EjLFY* expression for the same period (see Figure 2.1).

Different behaviour was observed for *EjAP1* expression. In this case, although no significant differences were detected between trees in mid and late July, in mid-August mRNA accumulation was 1000-fold lower in greenhouse trees than in field trees (Figure 2.8 B) coinciding with the result observed in the previous

year (see Figure 2.2). Consequently, no floral bud differentiation was found again under indoors conditions growing trees.

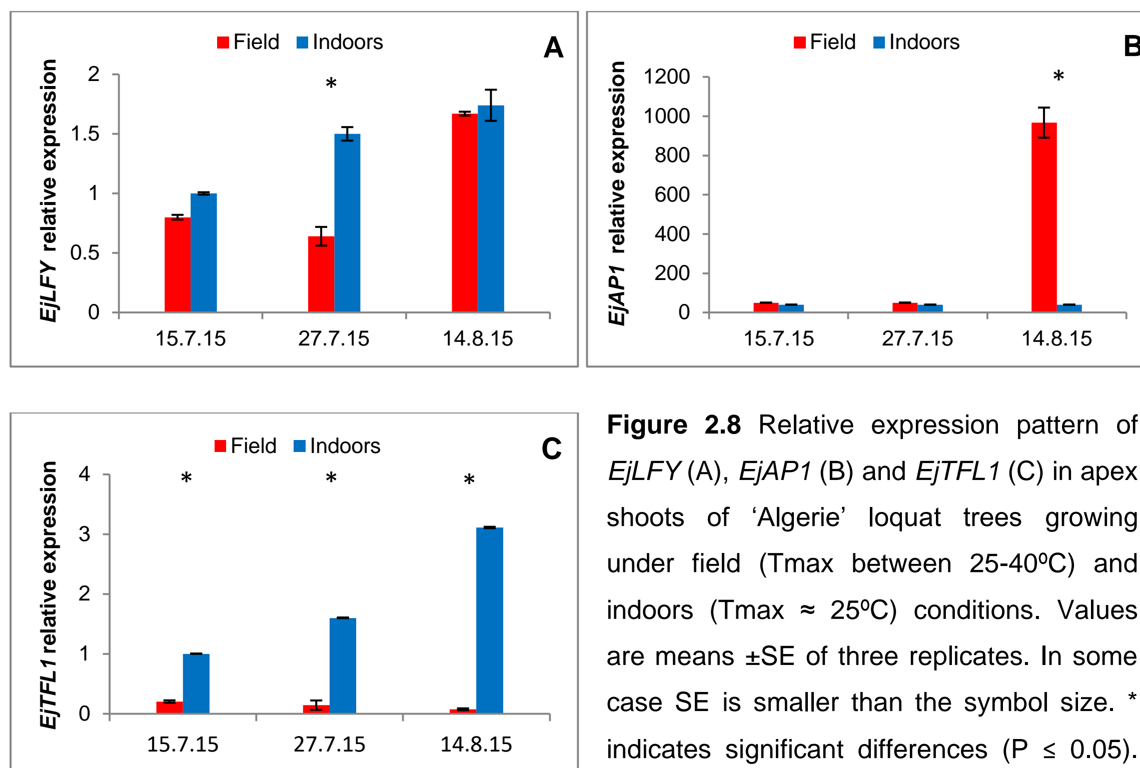


**Figure 2.8** Relative expression pattern of *EjLFY* (A), *EjAP1* (B) and *EjTFL1* (C) in apex shoots of 'Algerie' loquat trees growing under field (Tmax between 25-40ºC) and indoors (Tmax ≈ 25ºC) conditions. Values are means ±SE of three replicates. In some case SE is smaller than the symbol size. * indicates significant differences ($P \leq 0.05$). Values for 2015.

Regarding the floral repressor *EjTFL1* gene, significant differences were observed in transcripts accumulation in buds of the trees growing under field and indoors conditions for all dates studied, that in the latter being significantly higher than in the former (Figure 2.8 C). Interestingly, *EjTFL1* expression in buds of the indoors growing trees increased throughout the experiment reaching the highest level in mid-August when that for the field trees was the lowest (Figure 2.8 C). These results are partially in accordance with that observed in the previous year since the minimum *EjTFL1* expression in buds of trees under field conditions in mid-August coincided with the maximum *EjLFY* and *EjAP1* expression (see Figures 2.1, 2.2 and 2.3). Nevertheless, in buds of the trees growing indoors only the maximum *EjTFL1* expression in mid-August coincided with a low *EjAP1* expression (Figure 2.8 B and C), slightly differing with the previous year results (see Figures 2.1, 2.2 and 2.3).

Floral meristem identity genes expressions were also altered when trees were exposed to the indoors conditions during different periods along the summer months and early autumn.

In mid-July no differences were detected in the floral promoters expression genes, *EjLFY* and *EjAP1*, of terminal buds, regardless of the tree growing conditions, i.e, between A, B and C groups (indoors conditions) and D, E and F groups (field conditions) (Figure 2.9). The accumulation of transcripts of both genes was very low. Nevertheless, significant differences were observed in the floral repressor *EjTFL1* expression. In this date (mid-July) a higher expression of *EjTFL1* was detected in the indoors groups (A, B and C) than in the field ones (D, E and F) (Figure 2.9).

Fifteen days later, in late July, differences in *EjLFY* expressions were found between groups of trees grown under greenhouse conditions up to the time of sampling (B, C and D) and between those for field conditions (A, E and F). Nevertheless, whereas the accumulation of *EjLFY* transcripts was significantly higher in the former (2-fold in average) than in the latter, *EjAP1* transcripts levels were almost similar and unvaluable in all of them (Figure 2.9). It is important to note that trees of group D were transferred to indoors on 15$^{th}$ July, and only 15 days were enough to maintain the same gene expression (*EjLFY* and *EjAP1*) as groups C and B (33 days in the greenhouse). In a similar way as for *EjLFY*, in late July, transcript of *EjTFL1* accumulated mainly in apical buds of the trees under indoors conditions (groups B, C and D).

It is worth to notice how group D of trees, which were transferred to indoors conditions on 15$^{th}$ July, increased immediately *EjTFL1* expression maintaining a similar level to that of the trees of the group remaining indoors for a longer period as, C and B (33 days) (Figure 2.9).

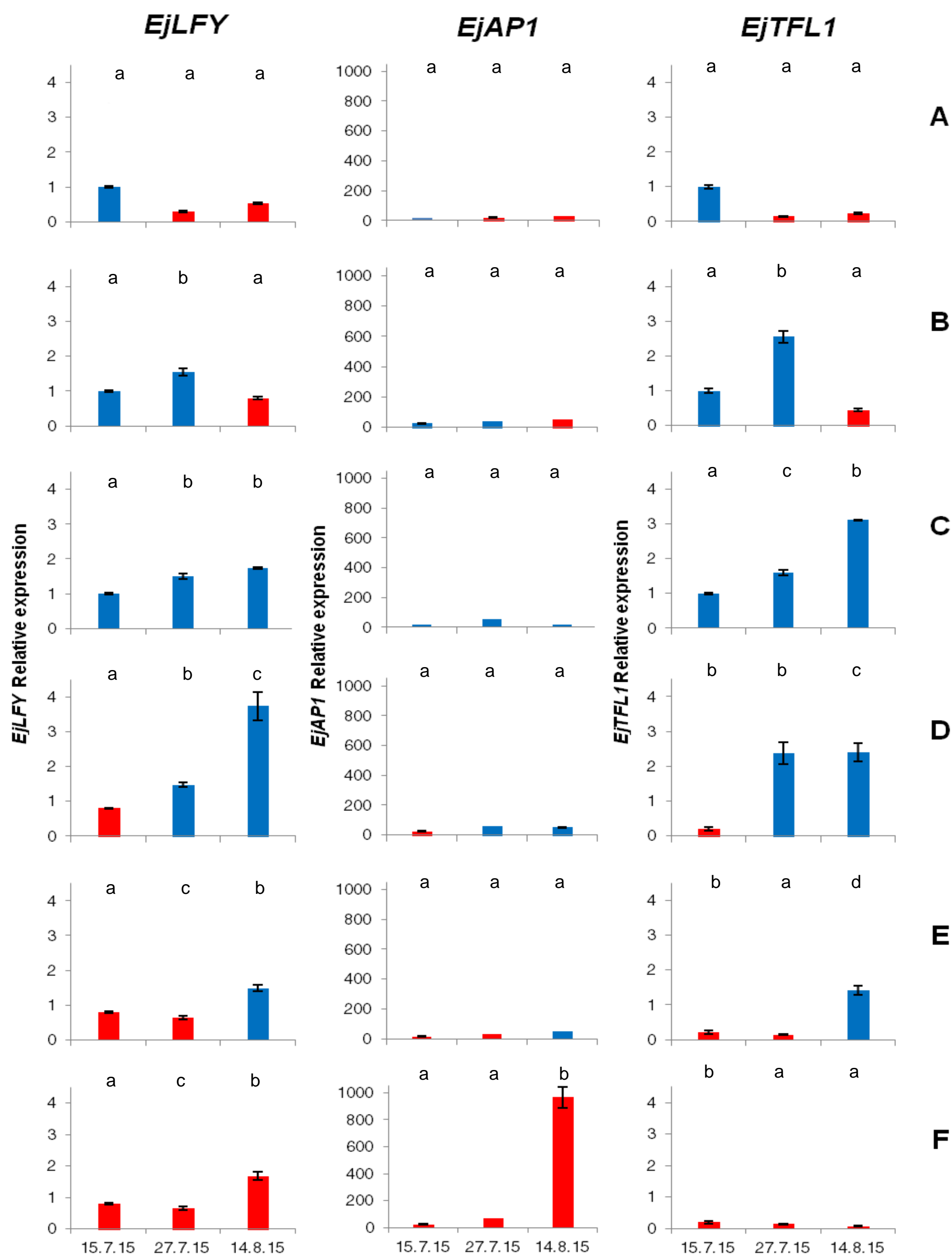


**Figure 2.9.** Relative expression pattern of *EjLFY, EjAP1* and *EjTFL1* in apex shoots of 'Algerie' loquat trees in different groups located indoors (Tmax ≈ 25ºC) from 25th June to 15th July (group A); from 25th June to 27th July (group B); from 25th June to 20th October (group C); from 15th July to 20th October (group D); from 27th July to 20th October (group E) and the field control (group F). Blue and red bars show trees located indoors and in the field, respectively. Data are means ± ES of 3 qRT-PCR replicates. Different letters for a given date mean significant differences (P ≤ 0.05). Values for 2015.

On the other hand, transcript of *EjTFL1* was downregulated in apical buds of trees grown under field conditions regardless the period remaining indoors. For instance, group A, which was kept indoors from 25$^{th}$ June to 15$^{th}$ July, displayed a level of *EjTFL1* similar to that of field control trees.

Eighteen days later, in mid-August, coinciding with floral bud differentiation period, high differences were found between the two floral identity genes, *EjLFY* and *EjAP1* expression. Thus, *EjLFY* expression maintained levels of expression similar to the previous sampling dates in all groups. Furthermore, group F did not show marked differences with regard to the other groups, being the buds of the trees from group D (indoors during this period) those with maximum level of *EjLFY* expression. But *EjAP1* expression in control field trees (group F) increased 1000-fold, being significantly higher than all the other groups. Consequently group F was the only one that flowered.

During this period, *EjTFL1* expression levels were also higher in the trees of the groups remaining indoors at sampling dates (group C, D and E). Group C (indoors conditions during the whole experiment) showed the highest expression. Besides, *EjTFL1* levels were similar in groups A, B and F. It is important to note how the group B, which late in July showed a high expression with respect to field control trees, on mid-August, after 18 days under field conditions, displayed a *EjTFL1* expression level similar to that of the field trees group (group F). On the contrary, group E increased its expression after being transferred indoors. In view of these results, average maximum temperature below 25 °C seems to upregulate *EjTFL1* expression.

The accumulation of *EjFT1* transcripts was significantly higher in the apical buds of the trees under indoors conditions than in those under field conditions in all dates studied (Figure 2.10). Thus, in mid-July (15$^{th}$ July) the former had a slightly higher expression (0.4-fold) than the latter. Fifteen days later (27$^{th}$ July) when group A and group D were transferred to the field and to indoors conditions, respectively, trees response to the thermal amplitude was relevant (Figure 2.10). Whereas in the trees of the group A *EjFT1* expression decreased significantly (0.8-fold) up to similar levels of the trees of the field groups (E and F), the trees of the group D also decreased the *EjFT1* expression, but only in 0.4-fold,

reaching similar levels of expression than in the trees of the groups B and C growing indoors. However, trees response to the temperature changes in mid-August was different. Twenty days after transferred trees of group B from indoors to field conditions, the accumulation of *EjFT1* also decreased, as for group A in the previous date, reaching a lower level than in the rest of the trees in the field (groups A and F).

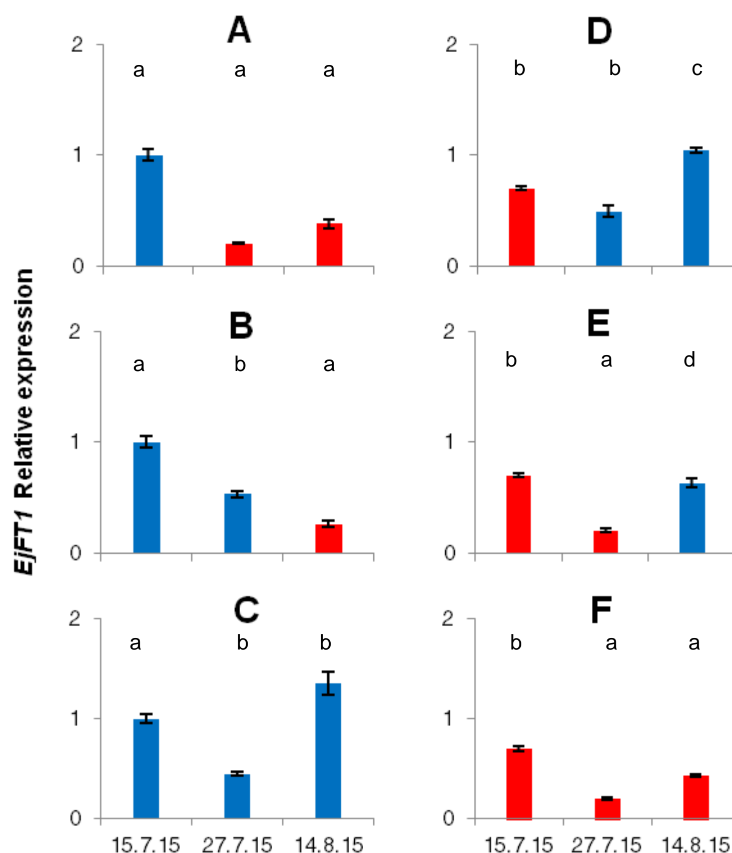


**Figure 2.10.** Relative expression pattern of *EjFT1* in apex shoots of ‘Algerie’ loquat trees in different groups located indoors (Tmax ≈ 25ºC) from 25th June to 15th July (group A); from 25th June to 27th July (group B); from 25th June to 20th October (group C); from 15th July to 20th October (group D); from 27th July to 20th October (group E), and in the field (group F). Blue and red bars show trees located indoors and in the field, respectively. Data are means ± ES of 3 qRT-PCR replicates. In some case SE is smaller than the symbol size. Different letters for a given date mean significant differences ($P \leq 0.05$). Values for 2015.

On the contrary, the trees of the group E which were transferred indoors on 27th July, increased in 1-fold the *EjFT1* expression in mid-August, reaching also a lower level of *EjFT1* transcript accumulation than the rest of trees growing indoors (groups C and D). In fact, the trees of group C, which were growing

under indoors conditions for the longest period, showed the highest *EjFT1* expression in mid-August when the floral bud differentiation takes place (Figure 2.10). Thus, indoors temperature conditions (average Tmáx 25ºC) could be an indicator of the floral bud transition stimulus perceived by the tree.

It is important to note that *EjFT2* expression was not detected in none of the group of trees.

Indoors temperature conditions (see Figure S2) also altered the ABA content in buds (Figure 2.11) as in the previous year (Figure 2.5). The abscissic acid concentration in the apical buds of trees growing indoors was significantly higher than in those of the trees growing in field, except on late of July when ABA concentration in the buds was similar in both trees (1200ng/ g DW) regardless of the growing conditions (Figure 2.11). Whereas ABA concentration in the buds of the trees growing indoors remained almost constant (1200 ng/g DW, on average), in field trees the maximum concentration (1136 ng/g DW) reached in late July (Figure 2.11).

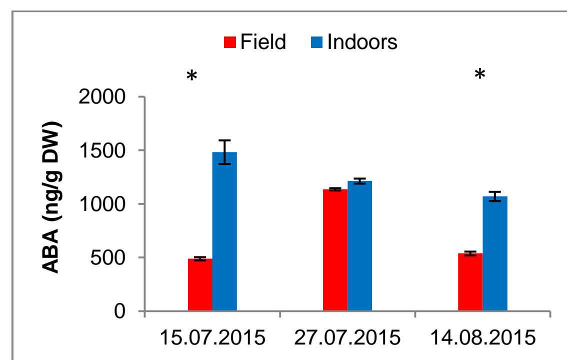


**Figure 2.11.** Time-course of ABA concentration in apex shoots of 'Algerie' loquat trees grown under field (Tmax between 25-40ºC) and indoors (Tmax ≈ 25ºC) conditions. Values are means ±SE of three replicates. In some case SE is smaller than the symbol size. Blue and red bars indicate indoors and field trees, respectively. * indicates significant differences (P≤ 0.05). Values for 2015.

Abscissic acid concentration in terminal buds was also significantly altered when trees (groups A-F) were exposed to the indoors conditions, i.e, average maximum temperature around 25 ˚C (see Figure S2), showing again a positive relationship between the temperature indoors and the ABA accumulation in the buds (Figure 2.12), as the previous year (Figure 2.5). On mid-July trees that

have been exposed to the indoors conditions for 20 days (groups A, B and C) showed a higher concentration of ABA (1500 ng/ g DW) than those exposed to field conditions (groups D, E and F) (500 ng/ g DW) (Figure 2.12). In fact, when trees were transferred from indoors to the field growing conditions, the ABA concentration in buds significantly decreased in a 65 % and in an 80 % for group A on 27th July and group B on 14th August, respectively (Figure 2.12).

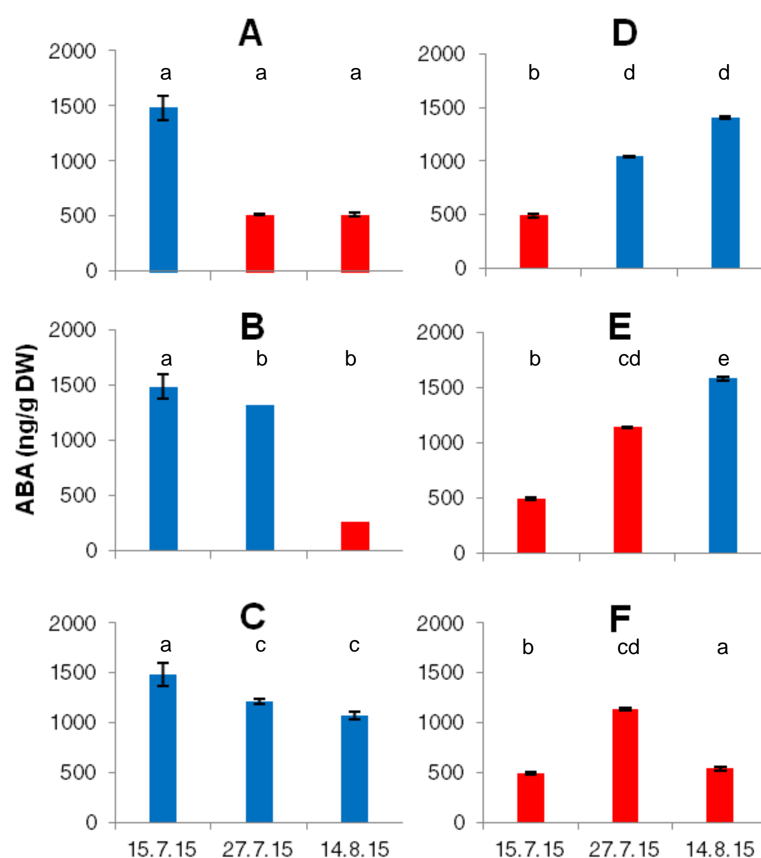


**Figure 2.12.** Time-course of ABA concentration in apex shoots of 'Algerie' loquat trees in different groups of trees growing indoors (Tmax ≈ 25ºC) from 25th June to 15th July (group A); from 25th June to 27th July (group B); from 25th June to 20th October (group C); from 15th July to 20th October (group D); from 27th July to 20th October (group E) and the field control (group F). Blue and red bars indicate trees growing indoors and in the field, respectively. Data are means ± ES of 3 qRT-PCR replicates. In some case SE is smaller than the symbol size. Different letters for a given date mean significant differences ($P \leq 0.05$). Values for 2015.

On the contrary, when trees growing in the field were transferred to the indoors conditions on the 15th of July (group D) and on the 27th of July (group E), the ABA concentration of their terminal buds increased, approximately, 20 days

later, in 552 ng/g DW (on 27th July) and 444 ng/g DW (on 14th August), respectively (Figure 2.12).

Nevertheless, not significant differences were found in all dates between tress growing indoors and field conditions in the gibberellins, auxins and cytokinins concentration of their terminal buds, as in the previous year (See Figure 2.6 and 2.7), indicating that these hormones are not related with the flowering response observed.

## 2.2. Identification of temperature heat requirements for loquat flower bud differentiation. A phenological model

A two-sequential phase's model is proposed to quantify heat requirements (HR) needed to flower. The first one quantifies the needs from floral bud induction to overcome floral bud differentiation; the second one those from floral bud differentiation to anthesis, only valid when HR fulfilling in the first one ends. Once sufficient heat is accumulated in buds during the summer, flowering takes places in autumn.

The empirical date (T0) to start HR quantification was forced to -190 day of the year (DOY), 24 June; the rest of parameters were left unforced.

The temperature response, *f* (T) more appropriated for the first phase is based on a non-linear ß function according to Wang and Engel (1998) version, using the cardinal temperatures, where $T_{max}$ (°C) is the temperature above which the development rate is zero, $T_{min}$ (°C) is the temperature below which the development rate is zero, *and* $T_{opt}$ (°C) is the temperature at which the development rate is optimal.

The temperature response, *f (T)* for second phase was based in Richardson function (Richardson *et al.,* 1974), where $T_{min}$ is the temperature below which the development rate is zero, and $T_{high}$ is the temperature above which the development rate does not increase anymore. The model parameters (Table 2.3) were estimated using the maximum air temperature data (first phase) and the average temperature (second phase) as input.

**Table 2.3.** Set of parameters estimated by the model

| | Phase one (Wang) | Phase two (Richardson) |
|---|---|---|
| Tmax | 36.39 | - |
| Topt | 33.14 | - |
| Tmin | 23.14 | - |
| Thigh | - | 46.76 |
| Tlow | - | 4.3 |
| SStar | 37.49 | 1252.98 |
| T0 | -190 (24 June) | -146 (7 August) |

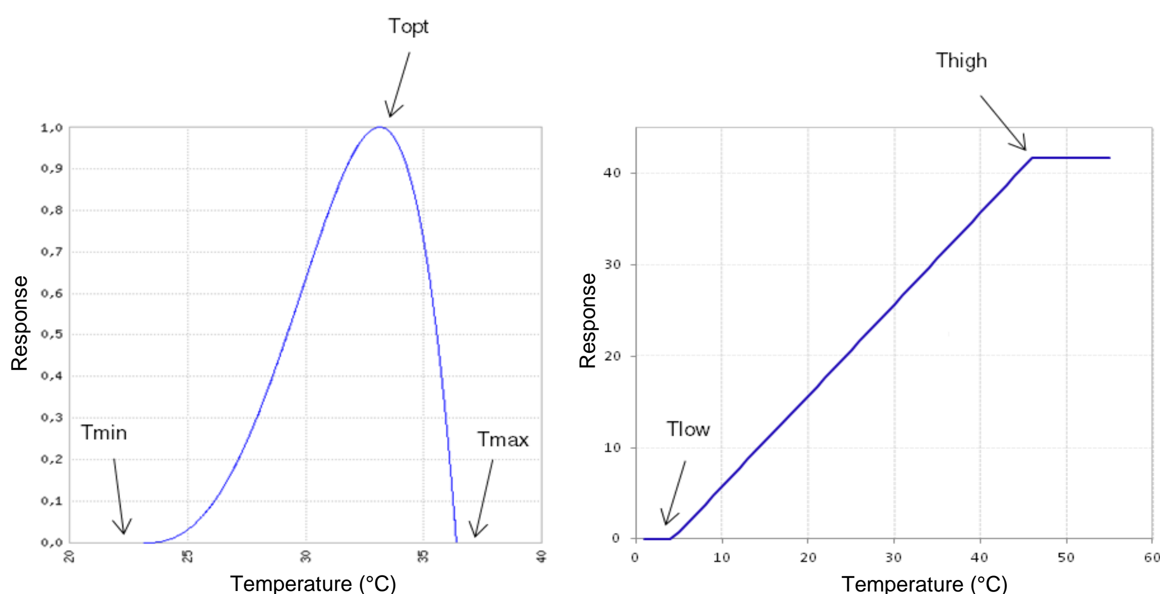


**Figure 2.13.** Temperature functions of a predicting flowering model using the Beta function (a) and the lineal function (b) for heat requirement calculation.

These functions (Figure 2.13) and parameters (Table 2.3) minimized the internal root means squared error RMSE. The model proposed had an efficiency of 0.43 and RMSE of 6.13.

The model predicts a date for floral bud differentiation (T0 of phase two) and a flowering date (end of phase two). Running a simulation with the climatic database, the model predicts a date for floral bud differentiation in the first half of August, but it would be necessary to have a register data of floral bud differentiation date to validate it.

## 3. Section 3. Floral bud differentiation: molecular and hormonal contents, heat requirement and microscopic analysis

Microscopic analysis of apical buds in August revealed first morphological changes in the meristem from vegetative to reproductive stage between the 8th and the 12th of August (Figure 3.1). On the 8th of August meristem did not displayed detectable changes to floral bud (Figure 3.1 A), whereas from the 12th of August onwards (Figure 3.1 B and B.1) the trimerous form, corresponding to first floral structures, was already detectable.

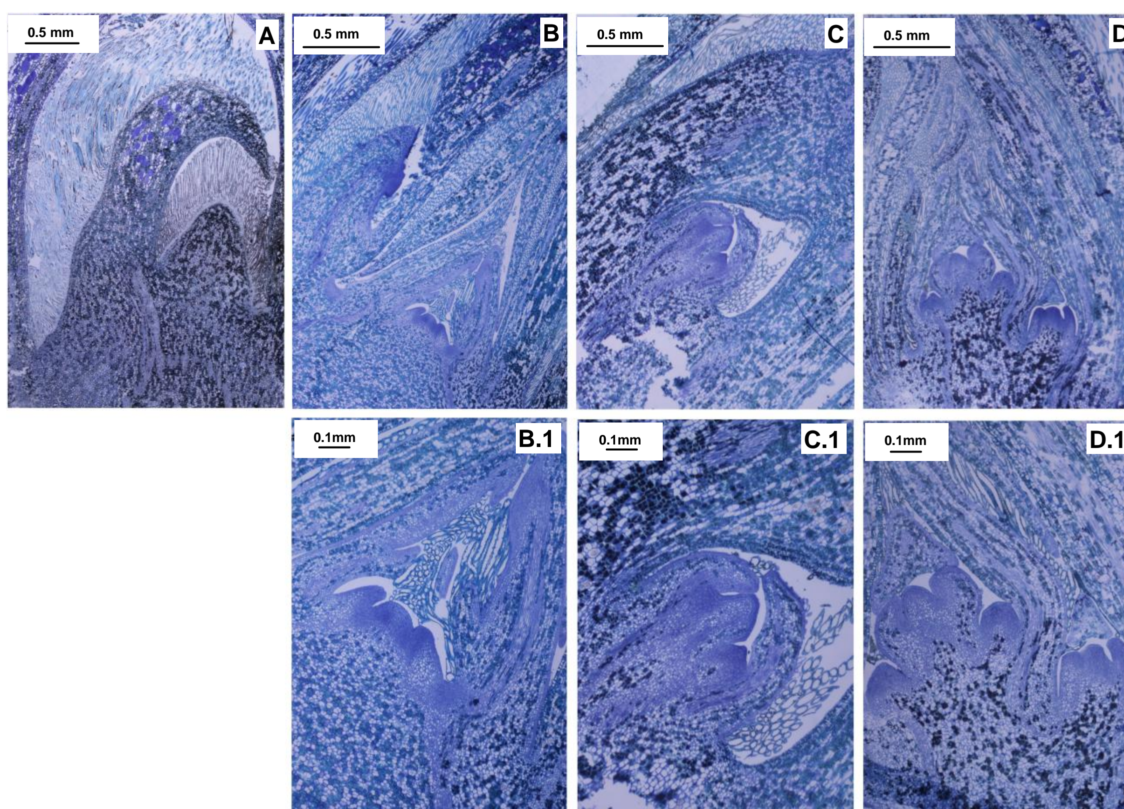


**Figure 3.1.** Flower initiation in 'Algerie' loquat. A) Vegetative apex corresponding to the 8th of August; B and B.1) 12th of August: trimerous forms corresponding to the first unequivocal floral structures; C and C.1) 16th of August: floral structures development are initiated; D and D.1) 19th August: floral buds show the begin of floral organs differentiation and outgrowth. Upper and lower plates differ in magnification.

*EjLFY* expression in the shoot apexes of 'Algerie' trees displayed an increasing trend from early August up to the 1st of September when reached a peak of expression (Figure 3.2 A). A similar time-course of expression was observed for *EjAP1* (Figure 3.2 B). From 1st of August its expression increased and reached a maximum level of expression on the 26th of August, 5 days earlier than *EjLFY.*

However, *EjTFL1* displayed the highest expression levels in late July reaching a peak on the 1st August; afterwards its expression dropped showing a continuous decreasing trend up to the end of the experiment (Figure 3.2 C).

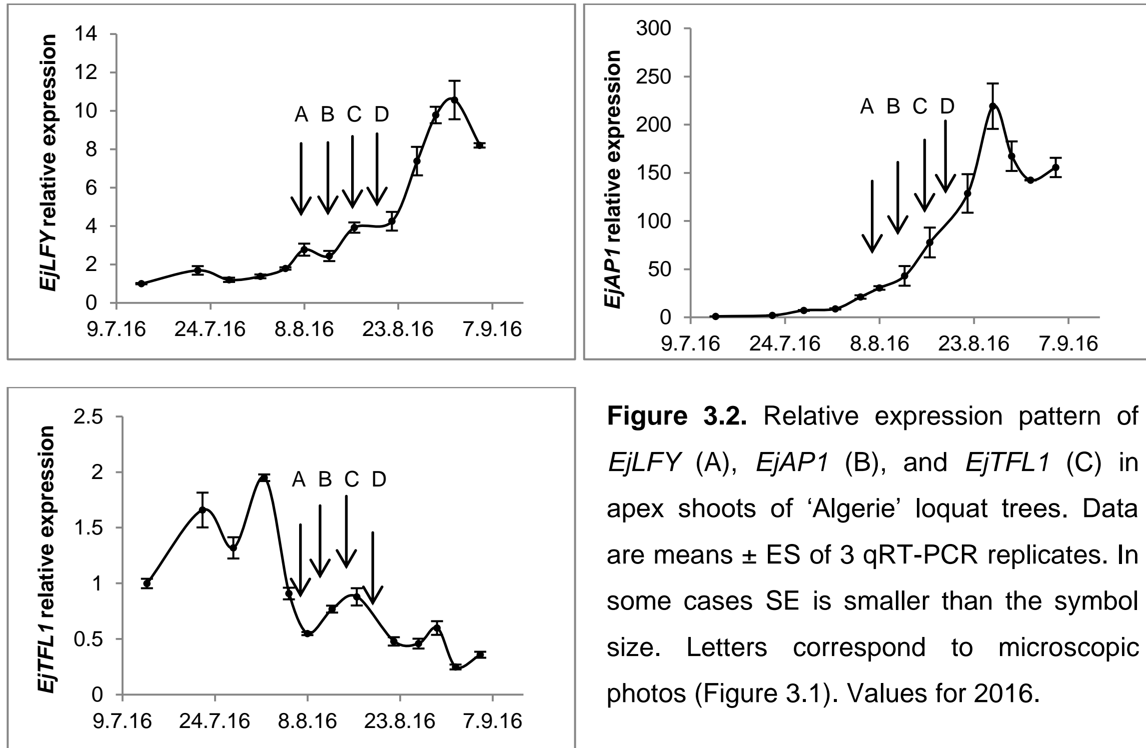


**Figure 3.2.** Relative expression pattern of *EjLFY* (A), *EjAP1* (B), and *EjTFL1* (C) in apex shoots of 'Algerie' loquat trees. Data are means ± ES of 3 qRT-PCR replicates. In some cases SE is smaller than the symbol size. Letters correspond to microscopic photos (Figure 3.1). Values for 2016.

The balance between promoters and repressor of flowering was reversed in the first half of August, coinciding with the period of the first morphological changes in the meristem (Figure 3.1), indicating that floral bud differentiation took place.

Time-course of ABA was similar to that of *EjTFL1* expression. ABA content in the apexes showed maximum concentration in mid-July (Figure 3.2). Afterward, it fell and remained low up to the end of the experiment, increasing slightly early in August.

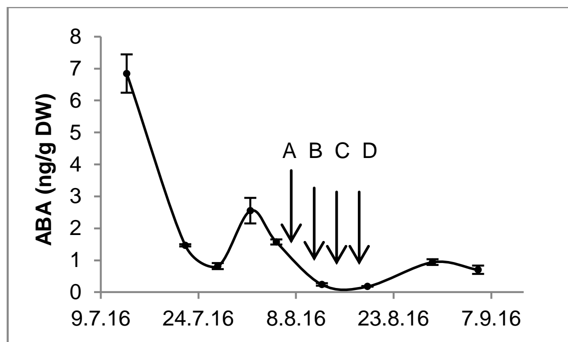


**Figure 3.3.** Time-course of ABA concentration in apex shoot of 'Algerie' loquat trees. Values are means ±SE of three replicates. In some cases SE is smaller than the symbol size. Letters correspond to microscopic photos (Figure 3.1). Values for 2016.

Applying the phenological model proposed in this study (Section 2.2), it predicted as floral bud differentiation date the $7^{th}$ of August, accordingly with the floral bud differentiation period indicated by microscopical and molecular analysis.

# *Discussion*

Plant growth and morphogenesis is controlled by meristems, organized tissues containing pluripotent stem cells whose identities and activities are regulated by intrinsic and environmental signals. The transition from vegetative to reproductive shoot meristem of most plant species is controlled by temporal and spatial interactions between external and internal factors, including environmental signals such as photoperiod and temperature, intrinsic growth regulators and a system of flowering genes. In Arabidopsis there are defined genetic pathways (photoperiod, vernalization, autonomous and gibberellin), which integrate the environmental and endogenous signals in deciding the timing of flower initiation. The acquisition of floral meristem identity (FMI) is controlled by the interaction of positive and negative regulators. Although several other genes have also been shown to play important roles in the regulation of floral meristem identity, the most important ones are *LEAFY (LFY), APETALA1 (AP1)* and *TERMINAL FLOWER1 (TFL1),* which seem to form the backbone of the network and, consequently, whose homologues have been studied in many other species.

In woody species, flowering process often extends to two consecutive seasons with a rest period in between – during the first season buds are formed and start the flower initiation that during the second season will develop and produce flowers or inflorescences (Wilkie *et al.*, 2008). Genetic analysis in perennials is a complicated task and, consequently, the function of the *LFY*, *AP1* and *TFL1* homologues from these plants is not as precise as in herbaceous species. Nevertheless, homologues have been analyzed in several perennial species and the available data indicate that these genes affect characteristics of woody plant development. As expected, expression of *LFY* and *AP1* homologues in perennials is also associated with floral bud differentiation whereas that for *TFL1* homologues is related to a repressor. Thus, in grapevine and apple expression of the *TFL1* homologues in developing buds is high during the initial stages of inflorescence development, but is absent later during flower development, when the expression of the *LFY* and *AP1* homologues becomes high (Wilkie *et al.,* 2008). The antagonism between the function of *LFY* and *TFL1* homologues, clearly shown by their generally mutual complementary expression patterns, seems to be generally conserved. Although they are often considered regulators

of floral identity specification, both genes seem to play a relatively general role in the control of meristem fate. Moreover, In Arabidopsis, *TFL1* is expressed at a low level in the shoot apical meristem (SAM) during the vegetative stage, while strong expression in the central zone of the inflorescence meristem represses *AP1* and *LFY* to maintain the indeterminate meristem (Liljegren *et al.,* 1999; Ratcliffe *et al.,* 1999). In *Arabis alpina*, a perennial relative of Arabidopsis, constant expression of *TFL1* in the SAM of young shoots prevents phase transition even after vernalization (Wang *et al.,* 2011).

Our results agree with this since during the flower bud differentiation period, *EjLFY* and *EjAP1* gene expression pattern is antagonistic to the *EjTFL1* gene expression, which is itself a signal that relays and coordinates shoot identity. Subsequently, *EjTFL1* may find its interactors to act regulating cell identity (Abe *et al.,* 2005; Wigge *et al.,* 2005). Moreover, functional characterization of *TFL1* in Rosaceae is confirmed as the major floral repressor which causes seasonal flowering, whereas mutation in this gene leads to perpetual flowering (Koskela *et al.,* 2012). In addition, the silencing of *TFL1* homologues in apple and pear also caused perpetual flowering in these species (Flachowsky *et al.,* 2012; Freiman *et al.,* 2012), suggesting that *TFL1* is a major repressor which contributes to the regulation of seasonal flowering within the yearly growth cycle in the Rosaceae. Koskela *et al.* (2012) proposed that flower initiation in *F. vesca* only occurs in autumn when *FvTFL1* is downregulated by SDs, whereas high *FvTFL1* in the spring may secure the production of new vegetative axillary shoots for the next growth cycle. In apple, when flower initiation occurs in early summer, *MdTFL1* is no longer detectable, and the expression levels of *MdAP1*, *MdLFY*, homologue and *MdFT*, are upregulated in the terminal shoot apex (Hattasch *et al.,* 2008; Mimida *et al.,* 2011a). At the same time, the SAM ceases to produce new leaf primordia, shoot growth stops, and floral differentiation begins. This is completely in accordance with the results of our experiments in which, for the both years, the minimum *EjTFL1* expression in the terminal buds, from late July onwards, coincided with the maximum *EjLFY* and *EjAP1* expression. Accordingly, microscopic studies reveal that the first indications of flower commitment are in late July (Rodriguez *et al.,* 2007), although flower bud differentiation takes place later, approximately in mid-August, as our

microscopically observations show. The first symptoms of flower bud differentiation in the terminal buds of the shoot were detected in mid-August ($12^{th}$) (see Figure 3.1). Nevertheless, it was suggested that high levels of initial *TFL1* mRNA may be needed to inhibit *LFY* effects and, therefore, to be a marker for a switch to bolting and inflorescences stages (Badley *et al.,* 1997; Ratcliffe *et al.,* 1999). That could be the reason why an *EjTFL1* accumulation is detected during late June and early July. In fact, taken together three rosaceous species tested so far, the maximum expression of *TFL1* mRNA is detected in the vegetative SAM, whereas its down-regulation precedes flower initiation. On the other hand, the function of *FT1* as a floral promoter in Rosaceae was confirmed, since silencing of *FT1* delayed the activation of *AP1* in the shoot apex and caused late flowering (Koskela *et al.,* 2012). This is consistent with the finding that the overexpression of *MdFT* in apple or *FT1* in plum causes precocious flowering (Kotoda *et al.,* 2010; Srinivasan *et al.,* 2012). Nevertheless, a recent modelling study suggests that *FT* may activate *TFL1* in the Arabidopsis inflorescence meristem (Jaeger *et al.,* 2013). Although further studies are needed to explore whether this occurs in the Rosaceae, the similar trend in the *EjFT1* and *EjTFL1* expression found in our experiments in the terminal buds of the trees grown in the field would coincide with this idea. An alternative hypothesis is that the strong repressor *TFL1* overrides the floral promoter function of *FT1*. This is possible since both *FT* and *TFL1* can bind *FD* transcription factor to control *AP1* in opposite ways (Hanano and Goto, 2011). Consistent with this idea, the previous *EjTFL1* accumulation observed in the terminal buds of the trees grown under indoors conditions during early July could explain the lower *EjFT1* expression detected in this date and why it increased later paralleling *EjTFL1* decrease. In addition, the FT/TFL1 ratio was shown to control flowering time as well as plant architecture in other species (Shalit *et al.,* 2009; Danilevskaya *et al.,* 2010). The following data indicate that down-regulation of *TFL1* homologues in rosaceous species correlates with vegetative to reproductive transitions during the anual cycle. In addition, the silencing of *TFL1* homologues in apple and pear caused perpetual flowering in these species (Flachowsky *et al.,* 2012; Freiman *et al.,* 2012). In other species, such as aspen (*Populus* spp), the expression of genes homologous to *FT*

correlates with growth cessation and bud set in addition to flowering (Bohlenius *et al.,* 2006; Hsu *et al.,* 2011). Moreover, the overexpression of *Populus FT1* in plum trees rendered them unable to enter dormancy (Srinivasan *et al.,* 2012), but the identification of *FT* as a mediator of temperature signal for growth cessation represented a milestone in the elucidation of the control of timing of flowering and seasonal growth cessation in trees (Bohlenius *et al.,* 2006). Despite the limited, but tantalizing, linkage between the floral regulatory machinery and seasonal growth cessation and bud set, it would coincide with our hypothesis, which suggests a general model for summer rest period regulation through high temperature required to induce flower bud differentiation in loquat. The higher *EjFT1* expression in buds of the trees growing indoors, i.e, trees whose apex inactivity by emitted leaves was not detected, than in field trees, would support it. Moreover, several studies have reported that the onset of dormancy begins at the end of summer, prior to arrival of chilling temperatures (Cook and Jacobs, 2000). In fact, Chuine and Cour (1999) documented that since summer temperatures could be related to the intensity of dormancy in deciduous Rosaceae species, an alternative devernalization mechanism, triggered by accumulated heat in summer, might be possible, as proposed by Battey (2000).

On the other hand, some rosaceous woody species genome, such as apple, contain several candidates genes homologous to Arabidopsis vernalization and ambient temperature pathways genes (Mouhu *et al*., 2009; Guitton *et al.,* 2011), such as *TFL1* which control temperature-dependent flowering. Therefore, the role of the recently identified ambient temperature-sensing mechanism (Kumar and Wigge, 2010; Kumar *et al.,* 2012) should be analysed in relation to the control of *TFL1* expression. According to this idea, the minimum level of *EjTFL1* transcripts found in our field trees from late July to late August could be related to the high temperature down-regulating *TFL1* even under previous inductive conditions. And opposite, the higher transcription levels registered under low temperature, i.e. indoors conditions, during the same period, impeding the bud to flower. In fact, environmental conditions exert some control over floral initiation in Rosaceae woody species. Temperatures below a certain threshold value impede the processes that lead to growth and prevent any external

indication of activity. This inhibition mechanism generally operates in all woody plants and seems to be caused by an endogenous factor of the meristem, present in both vegetative and reproductive buds (Champagnat, 1983) and with notable difficulties associated with the phenomenon. An importance advance was the establishment of different models as the Utah model (Richardson *et al.,* 1974), which assigned chill units values to different temperatures, or the Dynamic model, which established a synergic effect of moderate temperatures when combined with low temperatures (Erez and Couvillo, 1987). Several modifications to these models have been presented, such as the Low Chilling Model (Gilreath and Buchanan, 1981b), but all of them are only valid for a dormancy modelling. Nevertheless, loquat doesn't have a dormancy bud period associated with the unfavourable winter conditions, so new modifications to the models are needed to obtain solid conclusions about the suitability of the model, as it has been in this PhD. This method essentially consists of establishing the heat accumulation, above a threshold value, to which a loquat tree is exposed from breaking bud growth cessation during the summer rest period until flower bud differentiation and from flower bud differentiation to the anthesis. Crop-specific models for heat requirement and flowering dates have recently developed as a more specific approach in different countries as olive in Spain and Portugal (De Melo-Abreu *et al.,* 2004), apple in France (Legave *et al.,* 2008) and kiwifruit in New Zealand (Austin *et al.,* 2002). Although models are proxies for explaining flower bud differentiation overcoming with deep biological significance, this empirical model suggests that the biochemistry involved in the sensing of heat requirement and a predictive flower bud differentiation date is based on a functional understanding of tree physiology. In this context, the mentioned model predicts early August (7$^{th}$) as the flower bud differentiation date, approaching to the real date (12$^{th}$) observed with our microscopical studies. However, heat unit accumulation could not consistently account for the timing of floral initiation in 'Royal Gala' apple (McArtney *et al.,* 2001), but the temperature during the growing season can affect the intensity of floral initiation and it may be that temperatures which induce high vegetative vigour reduce floral initiation (Tromp, 1976, 1980). High root temperatures can inhibit floral initiation in lychee even when shoots are exposed to florally inductive temperatures (O'Hare,

2004), implicating either perception by the roots and long-distance signalling or heat transfer via the transpirational stream.

Previous studies indicate that plant hormones play an important role in the regulation of bud growth and flower induction, as well as in the growth and development of woody plants (Curaba *et al.,* 2014; Mutasa-Gottgens and Hedden, 2009). Plant hormones also take part in growth regulation in response to stress (Gazzarrini and Tsai, 2014), and also act as a signal molecules controlling growth and development through the regulation of gene expression, involving different pathways in response to environmental changes. mRNAs act as important regulators involved in multiple links with hormone crosstalk-mediating developmental processes, such as flower development, phase transition and stress responses (Curaba *et al.,* 2014). Studies in apple and pear indicate that hormone balance may control flowering in *Pyrinae* sp. In fact, the application of exogenous gibberelins (GA) inhibits flowering in some woody species (Bangerth, 2009), in contrast to Arabidopsis in which GA promotes flowering (Blázquez *et al.,* 1998; Li *et al.,* 2008). Although the relationship between GA and *MdLFY* remains unclear, the overexpression of *LFY* was not found to promote flower initiation in apple (Flachowsky *et al.,* 2010). Another possibility is that GA inhibits flowering through *TFL*-like genes (Roberts *et al.,* 1999). Our results disagree with that reports, since the time-course of the gibberellin content in terminal buds of loquat trees grown under field conditions, which were able to flower normally, and of trees growing under indoors conditions, which didn't flower anyone, was similar. Nevertheless, the time course of the ABA content between these trees was opposite, suggesting that low ABA content in buds correlates with normal flowering in loquat. ABA is a key regulator of seed development, dormancy, germination, and adaptive responses to abiotic stresses (Zeevaart and Creelman, 1988), and is involved in the regulation of floral and phase transitions in responses to environmental stresses (Gazzarrini and Tsai, 2014). Accordingly, it could be the reason why buds of the trees growing under indoors conditions, i.e. under stressed conditions, increase gradually the ABA concentration throughout the period studied, and prevent the flower bud to differentiate, which is enhanced by low temperatures. A high content of ABA in buds have been also related negatively with flowering in citrus

(Shalom *et al.,* 2014), and both endogenous ABA content and ABA application have been put forward as floral inhibitor via the downregulation of flowering genes (Martinez-Zapater *et al*., 1994, Blazquez *et al.,* 1998). In fact, reduction of ABA content anticipates *EjLFY* and *EjAP1* genes expression in our experiments, but we have not any evidence to correlate these two processes. ABA effect on flowering is still a dispute issue. In case of loquat, it might be a negative factor. Nevertheless, endogenous ABA also acts as a positive regulator of the drought escape response via the upregulation of the floral gene *FT* (Riboni *et al.,* 2013). Our results suggest that the decrease of ABA in the buds of the trees grown under field conditions could be a positive regulator signal for the floral bud differentiation process. On the contrary, indoors environmental conditions did not allow the decrease on ABA content, i.e. enhances its biosynthesis, thus impeding floral bud differentiation by interfering floral genes expression. Furthermore, apex of secondary shoots promoted by current shoot apex removal increased ABA content when removal was performed late in July, and it didn't develop into panicle, in contrast to those developed from earlier apex removal (early July), which developed flowers. Concomitantly, expression of *AP1* gene decreased, overall in the former, and prevented for flowering. Nevertheless, *LFY* expression increased for the latter. The lack of heat accumulation in the new apex forced to develop when current apex removal was carried out late in July agrees with our results, and it, together with increasing ABA content, might be reason why it didn't develop into flowers.

# *Conclusions*

Attending to the tested hypothesis in this PhD thesis the main findings are:

1. Temperature not exceeding 25°C reduced *EjLFY* and *EjAP1* genes expression and increased that of *EjTFL1* in the apex of loquat.

2. In loquat, the floral process seems not to be affected by endogenous gibberellin content since its time-course in terminal buds grown under temperature higher (field, flowers) and lower than 25°C (indoors, not flowers) conditions was similar.

3. The low level of *EjTFL1* transcripts found in our field trees from late July to late August might be related to the high temperature down-regulating *EjTFL1,* and opposite, the low temperature under our indoors conditions during the same period might be related to its higher transcription levels, impeding the bud to flower.

4. The decrease of ABA in the buds of the trees grown under field conditions could be a positive regulator signal for the floral bud differentiation.

5. Microscopically bud changes, ABA content, and *EjLFY*, *EjAP1* and *EjTFL1* genes expression allow to refer the second ten of August as the period of floral bud differentiation in loquat under Mediterranean climatic conditions.

# *Appendix*

## Appendix 1

**Table A1.** Fenological data of 'Algerie' loquat trees from Callosa and Palermo from 2004 to 2014

| Year | Growing area | Anthesis date (DOY) | Flowering data |
|---|---|---|---|
| 2004 | Callosa | 284 | 11-oct |
| 2005 | Callosa | 298 | 25-oct |
| 2006 | Callosa | 292 | 19-oct |
| 2007 | Callosa | 292 | 19-oct |
| 2008 | Callosa | 317 | 13-nov |
| 2009 | Callosa | 287 | 14-oct |
| 2010 | Callosa | 283 | 10-oct |
| 2011 | Callosa | 294 | 21-oct |
| 2012 | Callosa | 295 | 22-oct |
| 2013 | Callosa | 301 | 28-oct |
| 2014 | Callosa | 280 | 07-oct |
| 2004 | Palermo | 299 | 26-oct |
| 2005 | Palermo | 300 | 27-oct |
| 2006 | Palermo | 293 | 20-oct |
| 2007 | Palermo | 295 | 22-oct |
| 2008 | Palermo | 301 | 28-oct |
| 2009 | Palermo | 294 | 21-oct |
| 2010 | Palermo | 297 | 24-oct |
| 2011 | Palermo | 300 | 27-oct |
| 2012 | Palermo | 302 | 29-oct |
| 2013 | Palermo | 303 | 30-oct |
| 2014 | Palermo | 305 | 01-nov |

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