UNIVERSITÀ DEGLI STUDI DI PALERMO

DIPARTIMENTO DI SCIENZE AGRARIE E FORESTALI

Dottorato di Ricerca in Frutticoltura Mediterranea

FRUIT INHIBITS FLOWERING IN ALTERNATE BEARING CITRUS VARIETIES. HORMONAL, GENETIC AND EPIGENETIC REGULATION

SSD AGR/03 – Arboricoltura generale e coltivazioni arboree

Ph.D. CANDIDATE
Natalia Muñoz Fambuena

CO-SUPERVISOR
Dr. Domingo J. Iglesias Fuente

SUPERVISOR
Dr. Carlos Mesejo Conejos

CO-SUPERVISOR and Ph.D. COORDINATOR
Prof. Maria Antonietta Germanà

CYCLE XXVI - ACADEMIC YEAR 2015

DOTTORATO

UNIVERSITÀ DEGLI STUDI DI PALERMO

DIPARTIMENTO DI SCIENZE AGRARIE E FORESTALI

Dottorato di Ricerca in Frutticoltura Mediterranea

FRUIT INHIBITS FLOWERING IN ALTERNATE BEARING CITRUS VARIETIES. HORMONAL, GENETIC AND EPIGENETIC REGULATION

SSD AGR/03 – Arboricoltura generale e coltivazioni arboree

Ph.D. CANDIDATE
Natalia Muñoz Fambuena

CO-SUPERVISOR
Dr. Domingo J. Iglesias Fuente

SUPERVISOR
Dr. Carlos Mesejo Conejos

CO-SUPERVISOR and Ph.D. COORDINATOR
Prof. Maria Antonietta Germanà

CYCLE XXVI - ACADEMIC YEAR 2015

DOTTORATO

AGRADECIMIENTOS/ RINGRAZIAMENTI

Esta tesis ha supuesto un largo viaje que empezó hace casi 7 años, mucha gente ha influido en ella y por eso me gustaría agradecérselo. Una tesis implica mucho trabajo, que a veces no parece verse premiado, pero el resultado final compensa TODO.

En primer lugar me gustaría agradecer a Manuel Agustí el haberme abierto las puertas en su equipo en el IAM y haberme permitido trabajar y crecer cada día más, con sus consejos y su apoyo incondicional, sabiendo siempre como motivarme a seguir adelante. Cómo dice él, "No hay que rendirse nunca". Muchas gracias.

A mi director tanto de mi trabajo final de carrera, de tesina de máster y ahora finalmente de mi Tesis Doctoral, Carlos Mesejo. El cuál ha estado siempre a mi lado apoyándome y dirigiéndome en la dirección correcta. Además de no permitirme nunca desanimarme, levantándome en los momentos duros y siempre con una visión alegre y positiva de la vida. A ti gracias por todo.

A Carmina por transmitirme su buena actitud y consejos, apoyándome siempre en la consecución de mis objetivos. A Amparo, gracias, por ser mi amiga y por ayudarme siempre diciéndome y haciendo aquello que necesitaba en ese momento. A mis dos grandes compañeros, el ya doctor Roberto y a la futura doctora Ana, por su apoyo, alegría y amistad, esta tesis no habría sido igual sin vosotros. A Vicent por su buena disponibilidad siempre.

A todos los que han pasado por el IAM en estos años: Bartolomé, Giovanni, Caterina, Salvatore, Andrea, Nicola, Víctor, Fran, Alfredo, María, Diego, Alba, Marta, Alejandro, Helena, Tono. Quiero agradecer en especial a MªJosé y a Sara su ayuda y su buena actitud siempre. A todos ellos gracias por las risas y los buenos momentos en la Sala de Becarios. A mis compañeros doctores y futuros doctores, por esos almuerzos y comidas en las que hemos compartido tantas confesiones entre risas y siempre intentando ver el lado positivo de las cosas: Alex, Cristina, Altea, Juan Antonio, Vicky, David y Tono. A Mercedes, la cual siempre ha estado dispuesta a ayudarme con su alegría, buenos consejos y con la que he compartido grandes momentos.

Quiero agradecer del IVIA a Eduardo Primo, por animarme y enseñarme a investigar, permitiéndome aprender de su experiencia orientándome en la dirección correcta. También quiero agradecer a mi co-director Domingo J. Iglesias, que me ha apoyado siempre y del que he aprendido mucho durante esta tesis, demostrándome que la palabra 'confianza' existe en este mundo en el que trabajamos. Gracias a los dos por los buenos consejos. A Ana e Isa, mis 'mamis' durante mi periodo en el IVIA. Gracias por cuidarme, ayudarme y hacerme ver que todo tiene su lado bueno.

Del IBMCP quiero agradecer a Miguel Blázquez, por abrirme las puertas de su laboratorio y hacerme todo 'tan fácil'. A Paco, por su gran ayuda durante toda esta última fase,

gracias por tu buena actitud y disponibilidad siempre. A Aurelio Gómez y a Julián Cuevas, gracias por revisar y mejorar esta tesis.

A mis amigos, los cuáles han tenido que aguantar mis idas y venidas durante todos estos años….Gracias.

Questa tesi è stato un lungo viaggio che ha iniziato quasi sette anni fa, molte persone hanno influenzato e quindi vorrei ringraziare. Una tesi comporta un sacco di lavoro, che a volte non sembra essere premiato, ma il risultato finale vale TUTTO.

Mi piacerebbe ringraziare alla mia co-direttora Maria Antonietta Germanà per avermi aperto il suo laboratorio permettendomi di imparare a sviluppare una linea di investigazione ed appoggiandomi durante tutta la mia tesi dottorale. Grazie mille.

A Benedetta Chiancone, grazie, non solo per l'aiuto ricevuto durante il mio periodo italiano nel laboratorio, nel quale, i problemi non erano problemi. Ma soprattutto voglio ringraziarti che mi accogliessi nel tuo mondo, facendomi sentire come nella mia propria casa, essendo come una sorella maggiore per me.

A i professori Franca, Caruso, Marra, Paolo Inglese, Ricardo e Vittorio e a Laura, Roberto, Valeria, Germana, Fabrizio, Ahmed, Valeria G., Letizia, Marines, a tutti voi grazie per l'aiuto e le risate condivise. A Pina, per condividere con me la sua positività ed amicizia.

Alle mie ragazzine, Martina, Serena, Giulia e Rosalia per essere stato lì la mia famiglia. A mio fratello, Alessio, grazie per prenderti cura di me, aiutarmi sempre in tutto ed includermi nella tua vita. Grazie per includermi nel tuo gruppo di amici e che si trasformarono anche in miei, grazie a: Giuseppe, Francesco, Cristina, Alessia, Mariella, Giovanni, Alessia F., Luca, Emilio, Andrea.

Ai miei compagni e futuri dottori, per quelli momenti in cui abbiamo condiviso tante confessioni, ridendo e cercando sempre di vedere il lato positivo delle cose: Selene, Pino, Antonio, Fabrizia e Martina. In particolare, mi piacerebbe ringraziare a questo dottorato, l'avermi permesso di conoscere quella persona, la quale si è trasformata nella mia forza ed appoggio, e per la quale mi alzo ogni giorno, Walt.

Además, quiero agradecer a mis hermanos (Isa, Nuri, Mari, Nel-lo y Cris) a mis cuñados (Omar, Santi, Jaime y Lorena) y por supuestos a mis sobrinos (Jaume, Aless, Núria, Pau, Isa, Sami, Elena y Pep), el transmitirme siempre su energía, alegría y buena actitud siempre hacia la vida. Finalmente, a mis padres Manuel e Isabel, gracias por apoyarme, orientarme, aguantarme y quererme cada día. Os quiero, nada de esto sería posible sin vosotros.

"Haz que tú sonrisa cambie el mundo, no que el mundo cambie tú sonrisa"

Table of contents

Abbreviations

12ATX. TRITHORAX 1,2

5ATX. TRITHORAX 5

5-AzaC. 5-Azacytidine

7ATX. TRITHORAX 7

a/b. annual/biennial

ABA. Abscisic acid

AGL24. AGAMOUS LIKE 24

AGP. ADP-glucose pyrophosphorylase

AP1. APETALLA1

bZIP. Basic Leucine Zipper

C/N. Carbon/Nitrogen

CC. Companion cells

CcFLC. Citrus clementina FLOWERING LOCUS C

CDF1. CYCLING DOF FACTOR 1

CEN. CENTRORADIALIS

CETS. CENTRORADIALIS/ TERMINAL FLOWER 1/SELF-PRUNING

CG. Citosine/guanine

CHG.Citosine/ any nucleotide except G/guanine

CHH. Citosine/any nucleotide except G/any nucleotide except G

CiFT. Citrus unshiu FLOWERING LOCUS T

CKs. Cytokinins

CO. CONSTANS

CsLFY. Citrus sinensis LEAFY

CT. Control

DAT. Days after treatment

DEF. Defruiting

DNA. Deoxyribonucleic acid

ELF8. EARLY FLOWER 8

Exp. Expected

FD. FLOWERING LOCUS D

FKF1. FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1)

FLC. FLOWERING LOCUS C

FM. Floral meristem

FT. FLOWERING LOCUS T

GA. Gibberellin

GA20ox. Gibberellin 20-oxidase

GA_3. Gibberellic acid

GA3ox. Gibberellin 3-oxidase

GBSS. Granule-bound starch synthase

GFP. Green fluorescent protein

GI. GIGANTEA

GO. Gene Ontology

H3. Histona 3

H3K9. Histone H3 lysine 9

HP1. Heterochromatin Protein 1

HPLC. High-performance liquid chromatography

IAA. Indol acetic acid

IM. Inflorescence meristem

IP. Isopentyl adenine

JA. Jasmonic acid

LB. Lysogeny broth

LC/MS/MS. Liquid chromatography–mass spectrometry

LD. Long-Day

LFY. LEAFY

MADS. MCM1/AGAMOUS/DEFICIENS/SRF

MALDI, MS/MS. Mass spectrometry

MD. Malate dehydrogenase

MFT. MOTHER OF FT

MS. Murashige Skoog

MYB. MYELOBLASTOSIS

N. Nitrogen

NCED3. 9-cis-epoxycarotenoid dioxygenase

OFF. Without fruits

ON. With fruits

PBZ. Paclobutrazol

PCR. Polymerase chain reaction

PEBP. Phosphatidylethanolamine binding proteins

pI. Isoelectric point

PMF. Peptide mass fingerprinting

PPM. Plant Preservative Mixture

QTL. Quantitative trait locus

RNA. Ribonucleic acid

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

SAM. Shoot apical meristem

SD. Short-Day

SE. Sieve elements

SE. Standard error

SIM. Selected Ion Monitoring

SKB1. PROTEIN ARGININE METHYLTRANFERASE 5

SOC. Super Optimal broth with Catabolite repression

SOC1. SUPPRESSSOR OF OVEREXPRESSION OF CONSTANS 1

SPL3. SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3

SVP. SHORT VEGETATIVE PHASE

t-ABA. Trans-ABA

TEM1. TEMPRANILLO 1

TFL1. TERMINAL FLOWER 1

Theo. Theoric

TSF. TWIN SISTER OF FT

TSS. Total soluble solids

Tz. Trans-zeatina

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

VRN. VERNALISATION

X-Gal. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Abstract

In mature citrus trees, flowering is regulated by exogenous signals (i.e., low/high temperature, water shortage/water supply) that control flowering time, and by endogenous signals that control flower intensity. Actually, the ratio of endogenous promoters to inhibitors is considered as responsible for flowering; however, there is evidence suggesting that the inhibitors and their metabolism are the sole factors controlling flowering in *Citrus.* All the meristems have the information and ability to flower unless a negative factor hampers the process. The main endogenous factor controlling flower intensity is the fruit.

Attending to the aforementioned, in this PhD. thesis, the following hypothesis was tested:

Fruit inhibits flowering when ripening begins by exporting of hormones that induce epigenetic upregulation of flowering inhibitor genes in the leaves, and interfering in flower bud differentiation.

The main findings are:

1. The increase in *FLC* gene expression in ON-tree leaves coincides with fruit color change and low temperatures in the flower induction period. *CiFT2* is expressed in OFF-tree but not in ON-tree leaves.

2. The fruit produces and exports GAs and ABA to the leaves overlapping flower induction inhibition whereas these hormones decrease in OFF-tree leaves. GA_3 treatment reduces *CiFT2* gene expression but not *FLC* gene expression.

3. After bud sprouting, the inhibitory signals (*FLC, TEM1, SVP*) are not expressed in the new leaves. Every bud beside a fruit needs to restart vegetative growth to gain the flowering ability.

4. The fruit activates proteins from the stress responses and the oxidoreductase activity while in OFF-tree the primary metabolism and synthesis of starch is upregulated.

5. During the flower induction period, the fruit modifies the DNA-methylation profile of the *FLC* gene and increases the expression of methyltransferases that increase *FLC* gene expression. In defruited trees the process is reverted to the OFF-tree state.

Introduction

1. Flowering

Flowering comprises all of the development necessary for the irreversible commitment by the meristem to produce a flower or an inflorescence (Kinet, 1993). These stages include flower *induction*, flower *initiation*, flower *differentiation* and *anthesis*. The overall flowering process involves many steps, usually starting with perception of an environmental factor and terminating with the differentiation of three-dimensional structures, the floral primordia (Zeevaart, 1976). During development, plants must undergo a juvenile phase before transition to adulthood. A period of juvenility is characteristic to all higher plants. Juvenility was defined as the period during which a plant cannot be induced to flower (Wareing, 1959; Hackett, 1985; Poethig, 1990; Goldschmidt and Samach, 2001). This stage finishes with the first flower. During the juvenile phase of plant development, meristems acquire reproductive competence becoming able to sense and respond to signals that induce flowering. In annual/biennial plants this period is very short but in angiosperm trees it lasts for several years.

1.1 Flower induction, initiation and differentiation

In the adult stage, the plant is sensitive to inductive factors. *Flower induction* is the transition of the meristem from the vegetative to the reproductive phase. During this period, the leaf and the meristem receive flowering signals, and the genes required for flower development are turned on. As a result, nutrient, hormone and protein metabolism changes inside the bud. *Flower initiation* is the period when a series of histological changes are underway, but no visible morphological differences are observed. *Flower differentiation* is characterized by the development of the primordia of floral organs.

Chailakhyan (1937) proposed that a 'flower-inducing hormone' called *florigen* causes flowering. Movement of *florigen* inside the plant was demonstrated by means of grafting experiments. In annual/biannual plants this floral promoting factor is strong enough to even induce other plants as well if, for example, a single leaf is grafted onto a non-induced plant (Zeevaart, 1976), while in woody plants even though the inducing agent may be saturating a high number of the meristems always stay vegetative (Davenport *et al.*, 2006).

Searle (1965) stated that flower induction of photoperiod-sensitive plants is controlled by *florigen* that is produced in leaves and transported to buds. The export of the *florigen* in the short day plant *Perilla sp.* from the leaf to the shoot apical meristem was related with the idea of the long distance transport through the phloem (King and Zeevart, 1973). But the possibility of a slow transfer of the stimulus from cell to cell was also suggested from experiments with graft-induced plants where no vascular connections were formed (Wellensiek, 1970). *Florigen* was thought to play a positive role activating genes or a negative one, blocking gene-repressors.

After induction, the shoot apex changes from the vegetative to the flowering stage as a result of an increase in its 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).

1.2 When do plants flower? Exogenous and endogenous factors inducing flowering

Plants have developed the ability to detect seasonal changes in order to decide when to flower, according to their possibilities of development. Thus, the timing of flowering is regulated by autonomous and environmental factors, and four major pathways have been described to induce flowering: photoperiod, vernalization, gibberellin (GA) and autonomous pathways (Blázquez *et al*., 2006; Wilkie *et al.*, 2008).

Many flowering plants use photoreceptor proteins, such as phytochrome or cryptochrome, to sense seasonal changes in night length, or *photoperiod*. Long-Day (LD) and Short-Day (SD) plants flower in response to a change in the length of the dark period. LD plants flower when the night length falls below their critical photoperiod. These plants typically flower during late spring or early summer, as days are becoming longer. Examples of these plants are pea, barley, *Arabidopsis thaliana* or wheat. On the other hand, SD plants flower when the night length exceeds their critical photoperiod. These plants typically flower during summer and fall. Examples of these plants are cotton or rice. Facultative LD plants can flower under SD conditions whereas obligate LD plants cannot.

Neutral-day plants do not initiate flowering based on changes in photoperiod. Instead, they initiate flowering in response to alternative environmental stimuli depending on climate type. In temperate climates, a period of low temperature (*vernalisation*) induces flowering in many plants (e.g. tomato, rose, cucumber). When leaves are exposed to low temperatures, sugars accumulate and cold-regulated genes are induced to contribute to the cold acclimation process (Gorsuch *et al.*, 2010). Plants are able to detect the end of the cold period (winter) and, thus, flower in spring. In tropical climates, where no significant variation in photoperiod or temperature occurs, signals such as water shortage have the ability to induce flowering (Southwick and Davenport, 1986).

The hormonal (GA) and the autonomous pathways also induce flowering, and are largely independent from environmental influences (Parcy, 2005). In the autonomous pathway flowering is induced by internal cues at particular stages of plant development, whereas GA acts directly on the meristem at the flower initiation stage interacting with the sucrose content (Blázquez and Weigel, 2000). Therefore, the nutritional status also plays a role, at least as a source of energy, to induce flowering. In fruit trees a large number of buds remain vegetative in spring. This phenomenon was explained by a deficit of nutrients. Based on practical experience in fruit growing, the C/N-ratio hypothesis was used to explain differences in flower bud differentiation in fruit trees. A high C/N ratio favors flower formation and excessive N fertilization inhibits it. However, it seems that nutrients are not the limiting factor for flower formation when a threshold level is reached (Lang, 1965).

Finally, some of these factors interact to induce flowering. Studies of the photoperiodic responses of plants have demonstrated considerable diversity in the critical length of the dark period, the age at which seedlings are ripe-to-flower and the effect of temperature on photoinduction (Zeevart, 1976). In general, all wild populations of rice exhibit a strong SD response while cultivated varieties show a lower sensitivity (Katayama, 1971). Other plants, as sugarcane, require intermediate photoperiods, around 12 ½ hours, for floral initiation (Julien, 1973). The C_3 and C_4 pathways of photosynthesis also have been correlated with the photoperiodic requirement for flowering (Purohit and Tregunna, 1974). There is a group of plants originally considered to be strictly photoperiodic, which can also be induced to produce flower buds by factors other than daylength. One of these factors is the temperature. Thus, the

photoperiod requirements, besides the high or low temperatures, regulate flower formation (Blondon and Harada, 1972; Deronne and Blondon, 1973).

In the past, it has been very difficult to detect differences in metabolism or in a chemical composition between induced and non-induced plants, and therefore, results were difficult to interpret given changes in photoperiod or temperature. However, the development of genomic and transcriptomic tools has contributed to a better understanding of the metabolic and molecular processes involved in floral biology. Most of our knowledge about flower induction has come from studying flowering regulatory genes in *Arabidopsis thaliana*.

1.3 Floral induction in annual/biennial plants compared to mature fruit trees

Floral induction in mature fruit trees is distinct from that of annual/biennial (a/b) plants. In trees, it is a quantitative process with a significant proportion of the above ground meristems remaining vegetative, while in a/b-plants all the meristems are induced at once, which terminates the life of the plant (Bangerth, 2009). In a/b-plants, quantitative differences in flowering between species refer to the time of flowering during a given season (Zeevart, 1976).

In mature trees, reproductive competence varies between meristems, both apical and lateral, so that when they are exposed to favourable environmental conditions only competent meristems perceive flower inductive signals and differentiate into inflorescences and flowers (Walton *et al.,* 1997; Battey and Tooke, 2002; Martin-Trillo and Martinez-Zapater, 2002; Bangerth, 2009). Although there are differences between a/b plants and perennial plants, the genetics of flower induction and floral organ formation seems to be similar among them (Tan and Swain, 2007).

Bangerth (2009) presented two comparative models involved in floral induction of a/b and perennial plants: the *qualitative molecular-genetic model* and the *quantitative long-distance signal model* (**Table 1**). Both levels of regulation are at work in adult trees, and the expression of a number of *Arabidopsis* "floral integrator", "flowering time" and "flowering identity" genes, and orthologs of them, also occur in apple, citrus, grapes and other perennial trees and perennial herbaceous plants (Kotoda *et al.,* 2000; Pilliteri *et al.,* 2004b; Sreekantan *et al.,* 2004; Böhlenius *et al.,* 2006; Lifschitz *et al.,* 2006; Carmona *et al.,* 2007; Nishikawa *et al.*, 2007; Muñoz-Fambuena *et al.*, 2011).

However, this does not necessarily imply that these genes have the same or similar functions in trees and in *Arabidopsis*.

Table 1. Comparative scheme of two models involved in floral induction of annual/biennial- (left) and perennial (right) plants. Listed are decisive factors involved in the floral induction process. Obvious is the number of factors involved in FI in p-plants compared to a/b-plants (from Bangerth, 2009).

Qualitative "molecular-genetic" model	Quantitative "long-distance signal" model
Floral inducing cues	**Floral promoting cues**
Photoperiod: low temperature (vernalization): gibberellins; unknown autonomous factors stimulate flower induction	Exogenous factors: low temperatures (subtropical trees) (+); high light intensity (temperature fruit) (+); water shortage (some tropical trees) (+); high photosynthesis (sufficient carbohydrates) (+); production and transport of long-distance signals to accessible/non-accessible meristems
"Florigen" (FT protein): production and transport from leaves or other tissue to the Shoot Apical Meristem (SAM)	Endogenous factors: high number of fruits/seeds (biennial bearing) (-); strong vegetative growth (high GA concentration) (-); type of rootstock (strong/weak) (-/+); localization of buds along the shoot (accessible/non-accessible to floral promoters) (+/-)
Expression of flowering time/identity genes (morphogenetic transition)	Horticultural factors: optimal nutrition, particularly with nitrogen (+); partial removal of young fruit (thinning) (+); girdling, scoring (+); shoot bending (+); shoot pruning (winter/summer) (-/+); root pruning (+)
Floral morphogenesis	Hormones as long-distance signals: gibberellins (-); indoleacetic acid (-); cytokinins (+); ethylene (+?)
(+) promote floral induction; (-) inhibit floral induction.	

2. Molecular basis of flowering

Arabidopsis thaliana has proven to be an ideal organism to study plant development although it has no commercial value as it is considered a weed. During the past two decades, many flowering-related genes have been identified in *Arabidopsis thaliana*. Stimulators and repressors of flowering antagonize in metabolic pathways activating or inhibiting, respectively, the expression of genes that cause floral transition (**Figure 1**) (for more information see reviews by Blázquez *et al*., 2006; Wilkie *et al*., 2008; Amasino and Michaels, 2010; Andrés and Coupland, 2012; Pin and Nilsson, 2012; Blümel *et al*., 2015). This complex interaction of multiple pathways ensures the transition of a plant from the vegetative into the generative phase during favourable

environmental conditions. Genetic analysis of *Arabidopsis* flowering time mutants resulted in four major pathways controlling the time of floral transition: *photoperiod*, *vernalization*, *autonomous* and GA pathways. Whereas the photoperiod and the vernalization pathways mediate the response to environmental factors (light and temperature, respectively), the autonomous and GA pathways are endogenous (Martinez-Zapater *et al.*, 1994; Parcy, 2005; Wilkie *et al.*, 2008). Of those, the two main pathways promoting the expression of floral integrators genes in *Arabidopsis* are photoperiod and GA.

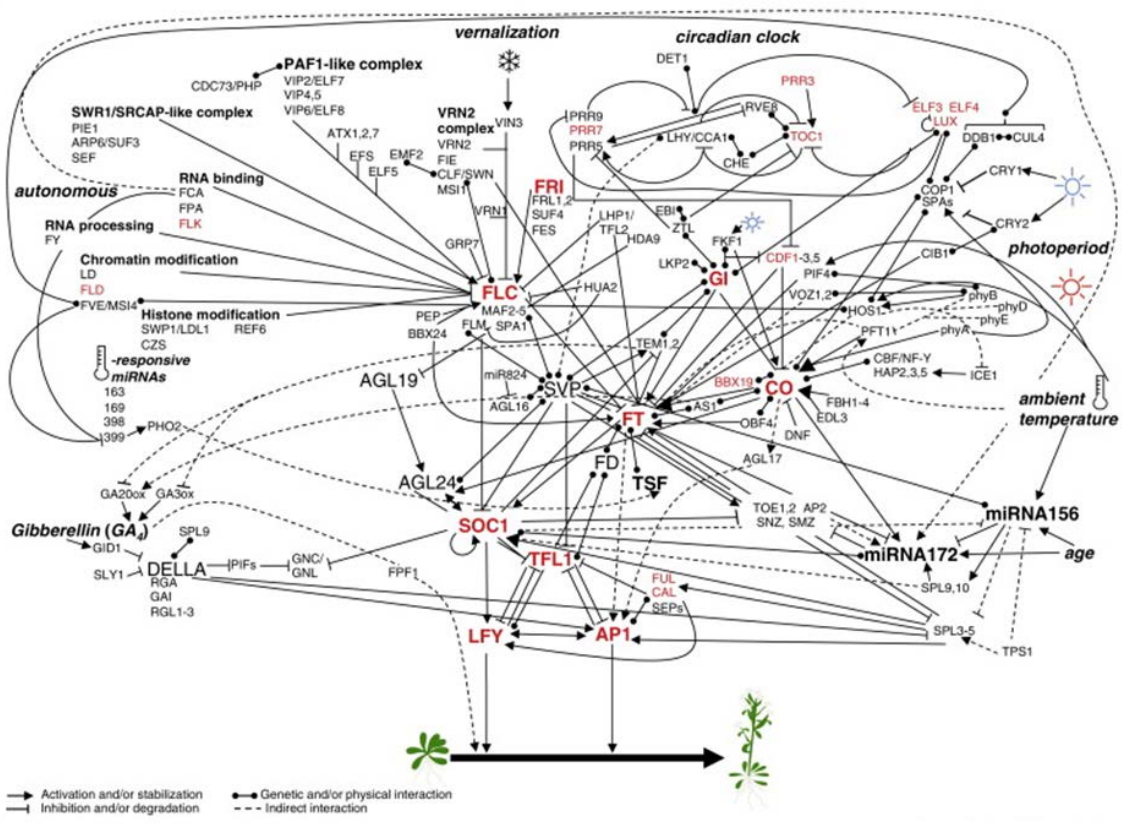


Figure 1. Flowering time gene network with known genetic and epigenetic regulators in *Arabidopsis thaliana*. Arrows indicate a promoting. T-ends indicate an inhibiting genetic interaction. Round dots at both ends mark an interaction without a known direction. Dashed lines denote an indirect interaction. Genes attributed as major regulators in the different flowering time pathways are written in bold. Red font indicates the functional characterization of a gene as a flowering time regulator in cultivated species – although not necessarily with the same function as in *Arabidopsis* – by mutant analysis, sequencing and complementation analysis or heterologous expression, RNA interference, or clear linkage with a major QTL. (From Blümel *et al.*, 2015).

2.1 Floral integrators genes and flowering pathways

Flower development occurs in the meristems, but floral induction occurs in the leaves. The spatial separation between these organs implies the need of a mechanism by which the floral signal is transferred from the leaf to the meristem. Among the network of genes implied in this mechanism *CONSTANS (CO), FLOWERING LOCUS C (FLC), FLOWERING LOCUS T (FT), SUPPRESSSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)* and *FLOWERING LOCUS D (FD)* play a main role in identifying and transmitting (or obstructing, in the case of *FLC*) the flowering signals, whereas *LEAFY (LFY), APETALLA1 (AP1)* and *TERMINAL FLOWER 1 (TFL1)* regulate the transition of the meristem.

Work in *Arabidopsis* has provided evidence that the small FT protein is (at least a component of) **the floral signal (*Florigen*)** transferred from the leaf to the meristem, and work in other species has strengthened this conclusion. *FT* gene expression is activated in the leaf and the derived protein is transported to the meristem (Kobayashi *et al.,* 1999; Michaels *et al.,* 2005; Teper-Bamnolker and Samach, 2005; Corbesier at al., 2007; Giakountis and Coupland, 2008; Jang *et al.,* 2009). The FT protein is a member of the *CETS (CEN1; TFL1; FT)* family (Pnueli *et al.,* 2001) and is related to phosphatidylethanolamine binding proteins (PEBP) family (Kardailsky *et al.,* 1999; Kobayashi *et al.,* 1999). In angiosperms, *PEBP* homologues are divided into three groups: *MOTHER OF FT (MFT), FT*, and *TFL1* (Kobayashi *et al.,* 1999). In *Arabidopsis, MFT* and *FT* gene families function promoting flowering, whereas the *TFL1* family delays flowering. 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) *Ipomea nil* (Japanese morning glory) (Hayama *et al.,* 2007), tomato (Lifschitz *et al.,* 2006), apple (Kotoda *et al.,* 2010) and citrus (Nishikawa *et al*., 2007; Muñoz-Fambuena *et al*., 2011). But FT protein is also reported to have nonflowering-related functions in some other species, namely tuberization in potato, and bud set in poplar and conifers (Böhlenius *et al.,* 2006; Rodriguez-Falcon *et al.,* 2006; Gyllenstrand *et al.,* 2007).

The transport of the FT protein from the leaf to the shoot apex is thought to be through the phloem (Corbesier *et al*., 2007) (**Figure 2**). The evidence for FT protein movement from the vascular tissue to the meristem comes from several experiments

achieved in different species. Experiments of fusion proteins such as FT::GFP expressed in phloem companion cells of *Arabidopsis* or rice were detected at the meristem, demonstrating their capacity for long-distance movement (Corbesier *et al.*, 2007; Tamaki *et al.*, 2007). Two mechanisms are proposed to elucidate as FT proteins enter the phloem sieve elements from the companions cells: diffusion of the FT proteins or active transport. Giakountis and Coupland (2008) proposed that the sieve elements of the phloem are made up of enucleate cells that are connected to each other at their end walls. When maturity is acquire, the sieve elements connect the photosynthetically active leaves to the growing parts of the plant (Oparka and Santa-Cruz, 2000; Zambryski, 2008). The sieve elements are closely connected to companion cells, which are nucleate. FT-mRNA is induced in these cells (Mathieu *et al.*, 2007). The companion cells are connected to the sieve elements by specialized branched plasmodesmata (Oparka and Santa-Cruz, 2000). Plasmodesmata act to facilitate the entry of macromolecules such as sugars, RNA, and proteins into the sieve elements. FT is smaller than the size exclusion limit of these plasmodesmata, and therefore could move into the sieve elements by diffusion. However, Giakountis and Coupland (2008) thought that since the protein is expressed at extremely low level in wild-type plants, a specific mechanism that permits the movement of FT into the sieve elements may be responsible for the movement of FT. Moreover, other authors observed that in *C.moschata* the movement of FT-like proteins into the sieve elements seems to be regulated by photoperiod, suggesting the involvement of a specific mechanism that can be influenced by photoperiod rather than diffusion (Lin *et al.*, 2007).

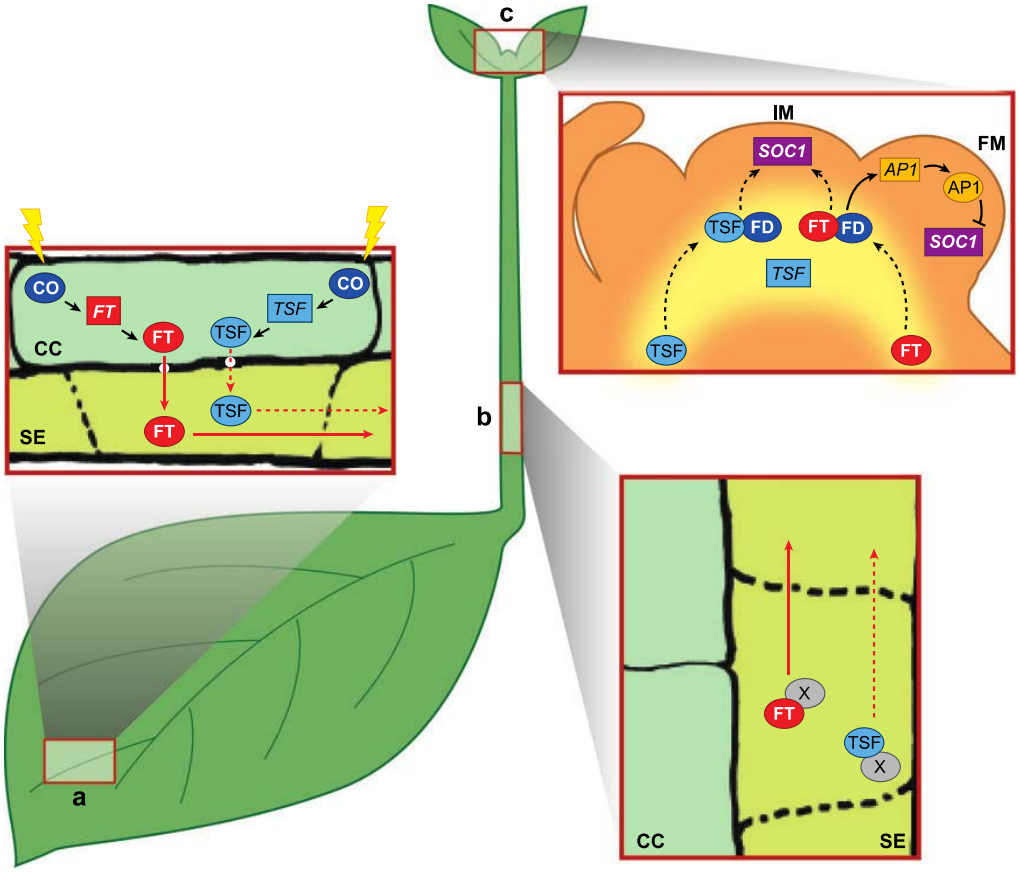


Figure 2. FLOWERING LOCUS T (FT) as a systemic signal. (a) CONSTANS (CO) protein allows for the transcription of FT and TWIN SISTER OF FT (TSF) in the phloem companion cells. FT protein is uploaded into the sieve elements either by diffusion through plasmodesmata or by an unidentified active transport mechanism (white circle). The similarity between FT and TSF proteins suggests they behave similarly. (b) Long-distance transport of FT toward sink tissues occurs in the phloem translocation stream. FT may associate with other as yet unknown factors (X) during this step. (c) FT unloading from the phloem and transport within the apex probably involves cell-to-cell transport through plasmodesmata. The yellow area indicates a possible gradient of FT and TSF protein distribution in the shoot apical meristem (SAM). Induction of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS (SOC1) is the first detectable event in the inflorescence meristem (IM) and it depends on the presence of FT and FLOWERING LOCUS D (FD). FT and FD interact physically and the complex is directly involved in APETALA 1 (AP1) transcriptional activation, which occurs during the formation of the first floral bud. AP1 directly represses SOC1 in the floral meristem (FM). CC, companion cells; SE, sieve elements; boxes, mRNA; circles, protein; solid black arrows, experimentally confirmed interconnection; broken arrows, inferred interconnection. (From Turck *et al.*, 2008).

The photoperiod flowering pathway is mainly directed via the expression of the gene *CO*. Exposure of plants to long day conditions results in a higher accumulation of the protein CO. Thus, the leaves should previously synthetize the RNAm of CO but it is only synthetized during the night and late afternoon. In fact, more RNA means more

protein, but this protein is stable only with light. For this reason the flowering in this type of plants is produced in spring-summer, when the high level of RNAm is formed, this moment coinciding with the maximum stabilization of the protein (Blázquez *et al.*, 2011). At when CO reaches this level, CO works as a transcription factor of the *FT* gene, and the related gene *TWIN SISTER OF FT (TSF)*, which are activated in the leaf by CO (Kobayashi *et al.*, 1999; Samach *et al.*, 2000; Michaels *et al.*, 2005; Teper-Bamnolker and Samach, 2005; Jang *et al.*, 2009). Fusions of the promoters of *CO* and *FT* to marker genes are expressed in the phloem, whereas the *FLAVIN-BINDING, KELCH REPEAT, F-BOX 1 (FKF1)* and *CYCLING DOF FACTOR 1 (CDF1)* genes, which encode regulators of *CO,* are also expressed mainly in this vascular tissue. Besides *CO, FHA* and *GIGANTEA (GI)* are activated in the rosette leaves of *Arabidopsis* in a circadian rhythm (Soltis *et al.*, 2002) under long day conditions.

The vernalization pathway of *FT* transcription is negatively modulated by other transcription factors. The MADS box transcription factors *FLC* and *SHORT VEGETATIVE PHASE* (*SVP)* directly bind to *FT* and repress its transcription (Searle *et al.*, 2006; Lee *et al.*, 2007). The *FLC* and *SVP* mRNAs are both expressed in the SAM as direct repressors of *SOC1* transcription (Lee *et al.*, 2000; Jang *et al.*, 2009; Liu *et al.*, 2009). *FLC* blocks *FT* 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. Cold is perceived in the shoot apical meristem by activation of the cold-response genes *VERNALISATION 1 (VRN1), VRN2* and *VRN3* and by changes in DNA methylation (Finnegan *et al.*, 1998). These factors suppress *FLC* expression; thus, vernalization promotes flowering by suppressing *FLC*. 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. *SVP* expression is reduced during the floral transition as *SOC1* mRNA starts to rise. So that soon after *SOC1* is strongly expressed in the meristem, *SVP* mRNA is not detectable (Albani and Coupland, 2010). The mechanism by which *SVP* is repressed in the meristem is unclear.

The GA pathway of flowering induction in *Arabidopsis* has little influence under LD conditions. But in SD, in the absence of the photoperiod flowering pathway, the GA pathway assumes a major role and becomes obligatory (Mutasa-Göttgens and

Hedden, 2009). GAs promote flowering in *Arabidopsis* through the activation of genes encoding the floral integrators *SOC1*, *LFY*, and *FT* in the inflorescence and floral meristems, and in leaves, respectively. In other LD species (i.e. *Lolium temelentum*) there is strong evidence that GA acts as a mobile signal to transmit the photoperiodic flowering stimulus (FT). While the role of GAs in flowering has become established for a limited number of species, GA is clearly not a universal flowering stimulus. In fact, it inhibits flowering in woody species. This paradoxical observation (GA acts positively in the switch to reproductive development in annual plants but negatively in woody species) was recently studied by Yamaguchi *et al* (2014). These authors found dual opposite roles of GA in *Arabidopsis* flowering promotion: GA promotes termination of vegetative development but it inhibits flower formation. To overcome this effect, the transcription factor *LFY* induces expression of a GA catabolism gene, and consequently, increases *LFY* activity causing reduced GA levels and flower formation.

2.2 FT-FD in the meristem and floral initiation genes

In the meristem, *FT* is believed to activate transcription of specific target genes by interacting with the bZIP transcription factor *FD* (Abe *et al.,* 2005; Wigge *et al.,* 2005). Both *FT* and *FD* are required for the upregulation of *SOC1*, but it is still not known whether this is a direct or indirect effect (Albani and Coupland, 2010). *SOC1* is an activator of floral initiation in the shoot apical meristem (SAM) and is upregulated soon after the shift from SD to LD (Giakountis and Coupland, 2008) or by GA (Moon *et al.,* 2003). Searle *et al.* (2006) showed that *SOC1* is expressed in leaves and shoot meristem, but it promotes flowering more powerfully when it is ectopically expressed in the meristem compared to the leaf. *SOC1* transcription is repressed by *FLC* (Hepworth *et al.,* 2002; Searle *et al.,* 2006; Sheldon *et al.,* 2006). Therefore, *SOC1* acts as an integrator of the photoperiod, GA, and vernalization pathways (Albani and Coupland, 2010). See **Figure 3**.

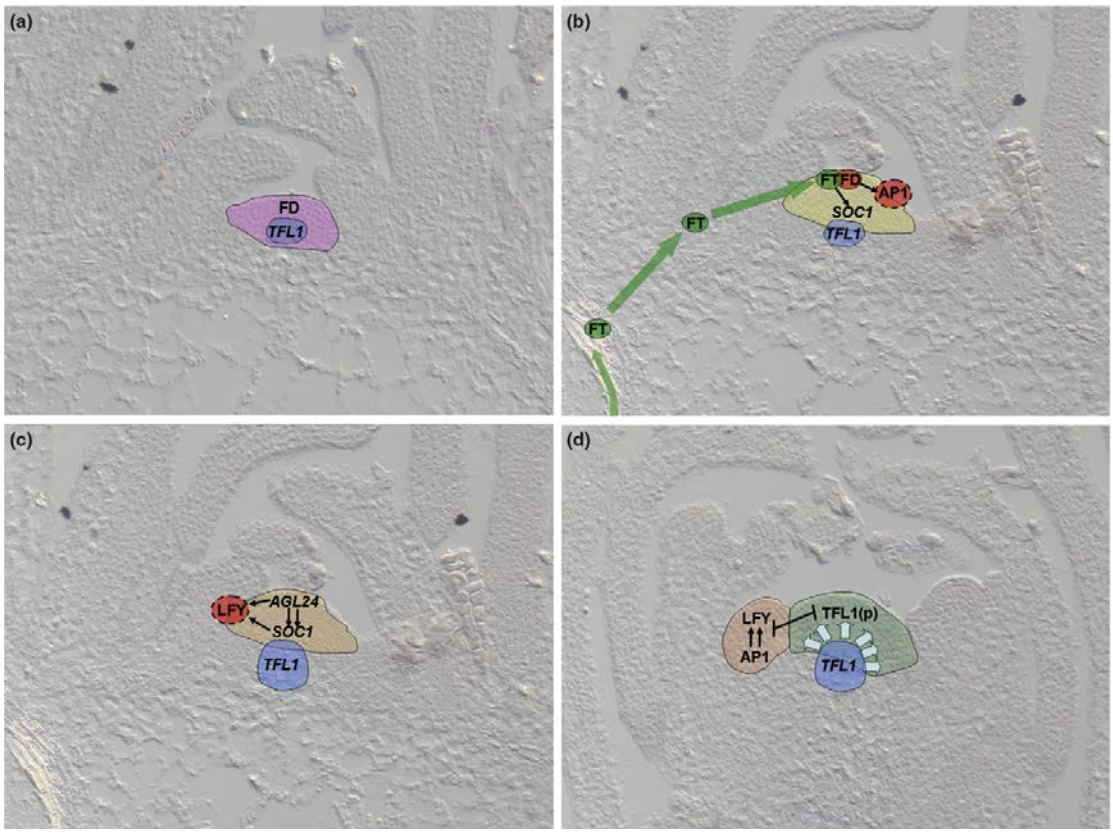


Figure 3. Spatial pattern of expression and molecular cascades associated with reprogramming of the floral meristem upon floral induction. (**a**) Vegetative meristem (no floral induction). *FD* mRNA (purple) is present in low amounts throughout the meristem while *TFL1* mRNA (blue) is expressed at low abundance in the center of the meristem. (**b**) Transition meristem (floral induction has occurred but no floral primordia are visible). Upon arrival from the phloem to the apex, the FT protein (green) interacts with the FD protein (red). This results in the direct upregulation of *SOC1* mRNA (yellow), one of the earliest known molecular markers of floral induction in the meristem. The FT–FD protein complex also upregulates, with a small delay compared to *SOC1*, *AP1* mRNA expression in the flanks of the meristem, in a region which will develop into a floral primordium (dashed red circle). (**c**) Floral committed meristem, early stage (floral commitment has occurred but no floral primordia are visible). *SOC1*, together with a gene called *AGL24* encoding another MADs box protein, participate in a positive feedback loop which eventually upregulates *LFY* expression in the flanks of the IM (Lee *et al.*, 2008) with a small delay compared to AP1. (**d**) Floral committed meristem (floral primordia are visible) TFL1 mRNA (blue) is now strongly expressed in the center of the IM while the protein (green) transports intercellularly (blue arrows) throughout the whole IM and represses LFY and AP1 transcription in the IM. At the same time both AP1 and LFY proteins (orange) ensure TFL1 does not accumulate in the floral primordia, separating these two features of the apical meristem. LFY and AP1 maintain their expression in the developing floral primordium through reciprocal upregulation. IM: inflorescence meristem. (From Giakountis and Coupland, 2008).

Among the floral identity genes *LFY* and *AP1* have the most significant influence. Both genes are expressed strongly in young floral primordia. Overexpression

of *LFY* or *AP1* are sufficient to confer floral identity to the SAM (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995; Jack, 2004). Strong *LFY* expression occurs rather late in the meristem during floral induction and is activated by several classes of transcription factors. Both *SOC1* and *SPL3* have been shown to bind directly to *LFY in vivo* using chromatin immunoprecipitation (Lee *et al.,* 2008; Yamaguchi *et al.,* 2009). Also, at least *in vitro*, the *AtGAMYB AtMYB33* transcription factors from the GA pathway bind directly to *LFY.* Activation of *LFY* by these transcription factors can explain why *LFY* acts downstream from several flowering pathways (Blázquez and Weigel, 2000). *LFY* directly activates *AP1* expression (Wagner *et al.,* 1999), and *AP1* can also provide feedback to activate *LFY* (Ferrandiz *et al.,* 2000). In this way *LFY* and *AP1* enhance each other's expression reinforcing floral identity and initiating floral organ specification and development.

2.3 Epigenetic control of flowering

Transcriptional control of a gene involved in flower induction is due, to a considerable extent, to histone modifications that are able to keep particular genes completely silent even in the presence of promoting signals. Floral induction in *Arabidopsis* has been studied in some detail with regard to histone modifications (Sung and Amasino, 2004). Nelissen *et al.* (2007) show that ATP-dependent modifications of core histones in the nuclei of cells have a great influence on the accessibility of the DNA of particular genes to transcription factors or the whole transcriptional complex. In brief, histones consist of four types of constitutively present proteins onto which the DNA is tightly-packed (heterochromatin). In this configuration, DNA is transcriptionally inactive and, therefore, non-accessible and not transcribed. However, signals reaching the cell surface can rapidly activate physiological mechanisms in the cells that covalently modify core histone tails, making DNA accessible (euchromatin) for the binding of transcriptional factors. These covalent modifications of core histone tails affect almost all aspects of plant development, including flower induction (Nelissen *et al.,* 2007). The physiological mechanisms needed to accomplish these modifications are methylation, acetylation, phosphorylation, and ubiquitination, together with their respective enzymes, which modify particular amino acids in the "tail" of the histone, thus causing conformational changes resulting in a loosening or tightening of DNA-histone binding. These methylation or acetylation patterns among

the histone proteins are partially inherited and, due to changes in the former terminology, considered as epigenetic and as an additional order of regulation not involving the DNA code.

While methylation of a particular histone tail often represses gene transcription, acetylation usually has the opposite effect and promotes DNA transcription. The acetylation and de-acetylation of histones are readily reversible enzymatic processes and enable increased transcription in addition to complete silencing of particular genes (Nelissen *et al.,* 2007). Among the examples that have been investigated more intensively for floral induction is the cool treatment induced of *Arabidopsis* and other plants (vernalization) caused by an epigenetic silencing of the gene *FLC*. This silencing is due to the histone methylation of *FLC,* which reduces its repressing effect on downstream genes, leading to earlier flowering. However, more detailed studies have shown that this process is more complicated insofar as it is accompanied by the de-acetylation of the histone H3, whose stable epigenetic repression is executed by the methylation of the histone HP1 (Bastow *et al.,* 2004). This histone modifies another histone, H3K9, which is involved in heterochromatin formation and, thus, in the down-regulation of *FLC* activity (Mylne *et al.,* 2006). Vernalization considerably increases the activity of a number of genes (Bäurle and Dean, 2006), some of which are known to code for transcription factors involved in histone modifications and ultimately in floral induction.

Aside from vernalization, light-dependent floral induction has been examined with regard to histone modifications and chromatin modeling/re-modeling. Studies into light duration effects in *Arabidopsis* demonstrated that light (similar to vernalization), gibberellins, and the autonomous pathways use the *FLC* gene to regulate floral induction (Takada and Goto, 2003; Boss *et al.,* 2004; Deal *et al.,* 2007). In these light processes histone modification and cromatin re-modeling were again involved. However, in contrast to vernalization, these histone modifications were different for all four of the previously mentioned floral induction pathways.

An alternative regulatory possibility for the silencing of genes relevant to floral induction is the post-transcriptional action of microRNAs (miRNAs), which also seem to interfere with the action of genes like *FLC* (Schmid *et al.,* 2003).

3. Flowering in fruit tree species

Fruit tree species show two main characteristics in relation to flowering: 1) a long juvenile phase (*juvenility*) of trees propagated by seeds, during which all the meristems in the tree cannot be induced to flower and remain vegetative; and 2) flowering is a quantitative process during the mature stage, with a significant proportion of the aboveground meristems remaining vegetative or dormant. This mechanism guarantees a long life span for these plants. Very little is known about how a tree achieves this trait.

The long juvenile phase is a major problem in breeding programs but not in fruit production, because fruit trees are propagated by grafting and thus are in their mature stage from planting. During the mature stage, fruit tree flowering seems to be rarely associated with photoperiod and vernalization (Wilkie *et al.,* 2008). In many subtropical and tropical tree species, such as mango, lychee, macadamia, avocado, and orange, flowering is induced by cool temperatures. The temperature required for flowering in such trees is between 10ºC to 15ºC and are higher than those required for vernalization in herbaceous species. When cultivated under tropical conditions, water shortage also induces flowering. Unlike subtropical and tropical trees, flowering is regulated autonomously in many temperate deciduous tree species, such as apple.

Kobel (1954) reported that specific factors inducing flower bud development are concentrated in the appropriate leaves attached to the bud. According to Bünning (1952), the developmental stage of the leaves is essential for the formation of flower organs. **The role of leaves** in the formation of flowers can be explained in three ways: 1) the leaf, as an organ of assimilation, provides carbohydrates needed in flower induction, 2) the leaf is a major site of hormone synthesis and 3) the leaf is the receptor of environmental signals. In the post-genomic era when genes have been identified as controling floral development, it became evident that important floral-inducing genes are active in leaves. It should also be mentioned that defoliating leaves before flower induction can greatly inhibit flower formation in apple (Li *et al.,* 1995) and citrus (Muñoz-Fambuena *et al.,* 2012b). The stage of flower induction was studied by removing leaves at different times before and after full bloom in several species (Fatta del Bosco, 1961; Li *et al.,* 1995). When defoliation has no inhibitory effect on flowering, the implication is that meristems have passed the flower induction period.

But the most outstanding point, and common to most fruit tree species, is the **inhibitory effect of fruit** on flower induction, even with the presence of exogenous flowering signals. In most species, fruit development coincides with the time of flower induction. Chan and Cain (1967) demonstrated that the presence of seeds is crucial in fruit inhibition of apple flower induction. But in parthenocarpic citrus species (Martínez-Fuentes *et al.*, 2010; Muñoz-Fambuena *et al.*, 2011), the fruit also inhibits flower induction, so the mechanism must not be exclusively attributed to seeds. The fruit inhibits flowering from the time it completes its growth, not before, and starts ripening. The process has been observed in orange (Martínez-Fuentes *et al.*, 2010), mandarin (Muñoz-Fambuena *et al.*, 2011), loquat (Reig *et al.*, 2014), olive (Dag *et al.*, 2010) and avocado (Ziv *et al.*, 2014).

The inhibition of flowering induces ***alternate (biennial) bearing***, a critical horticultural problem characterized by large yields of small-sized fruit in the "on-year", and low yields of oversized fruit in the "off-year" (Handschack and Schmidt, 1990). This is caused by the adverse relationship between fruit development and flower bud induction or differentiation (Monselise and Goldschmidt, 1982). Genotype is probably the major cause for alternative bearing. Within a given species, there are regular bearing cultivars and cultivars whose trend is to be very biennial bearing (**Fig. 4**).

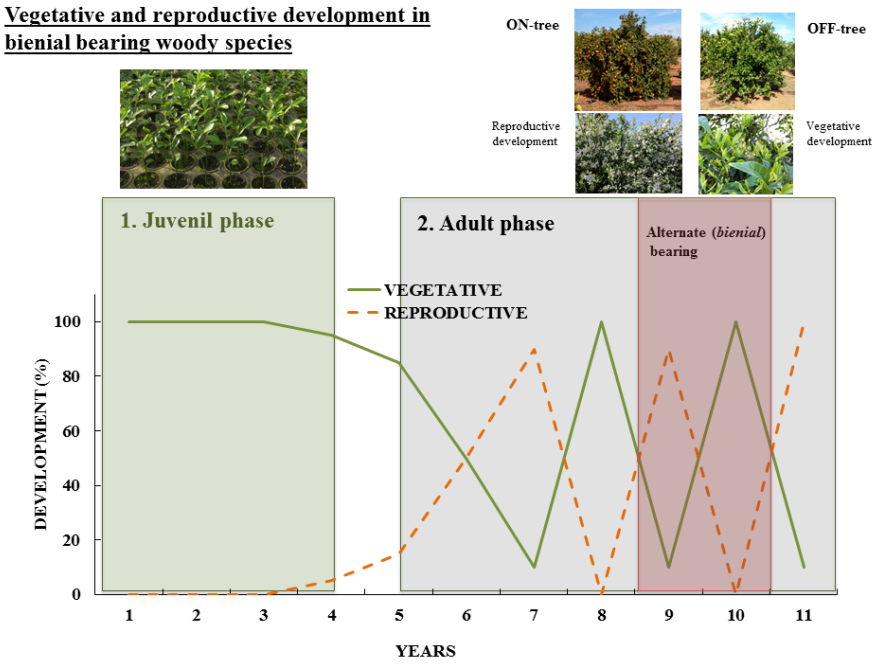


Figure 4. Schematic representation of the vegetative and reproductive development of biennial bearing woody species

Besides, **gravimorphism** plays an essential role in fruit trees. Vegetative growth is affected negatively by gravity (Smith and Wareing, 1964). Shoot growth and flower bud formation is obviously affected by shoot orientation, namely placing shoots in a horizontal position increases flower-bud formation and reduces growth (Longman *et al.*, 1965). Tromp (1982) found that shoot growth was reduced and flowering was increased by shoot bending and by application of a chemical growth inhibitor. In all cases a promotion of shoot growth increases apical dominance and *vice versa,* an inhibition of shoot growth supports lateral flower-bud formation.

The **position of an axillary meristem** on the plant, its age/size, and time of outgrowth might also determine its developmental fate. Vegetative development in apple and *Arabis alpine* is maintained by axillary meristems close to the SAM (Foster *et al.,* 2003; Wang *et al.,* 2009). Additionally, in kiwifruit the size of the meristem prior to bud break in the spring can determine the fate of the second-order axillary meristems (Walton *et al.,* 1997).

Flowering probability also depends on both **shoot and tree age**. In some pome fruit species, newly developed long shoots have a lower flowering ability than the older shoots. There is a decline corresponding to a growth reduction and an increase in the probability of flowering from the center of the tree towards the periphery. This centrifugal gradient has been found in certain tree species (Costes *et al.*, 1992; Sabatier and Barthelemy, 2001). However, other species such as citrus, the younger shoots sprout and flower with a higher intensity than the older ones (Agustí, 2003).

According to Bangerth (2009), two levels of flowering regulation act at once during flower induction in fruit trees: the *qualitative molecular-genetic regulation* and the *quantitative long distance signal regulation*.

3.1 Qualitative molecular-genetic regulation of flower induction in trees

In fruit tree species, the expression of flowering genes orthologous of *Arabidopsis* have been studied in relation to the specific signals which inhibit or promote flowering. A number of these have been identified in non-related species, which suggests a certain degree of evolutionary conservation. For instance, the expression of the *FT* gene has been identified in apple (Kotada *et al.,* 2010), mango (Nakagawa *et al.,* 2012), avocado (Ziv *et al.,* 2014), orange (Muñoz-Fambuena *et al.*, 2012a), mandarin (Nishikawa *et al.,* 2007), poplar (Böhlenius *et al.,* 2006), etc. In this

Ph.D thesis, species from the genus *Citrus* were the model trees selected to study the inhibitory effect of fruit on flowering. Therefore, this section of the reviewed literature focuses on studies conducted with *Citrus sp*.

In Citrus, the flowering-related genes characterized in *Arabidopsis* seem to be conserved (Dornelas *et al.,* 2007). For this reason, on the basis of data from *Arabidopsis*, citrus homologues of *FT, LFY, AP1, TFL1* and *SOC1* have been identified and characterized (Kobayashi *et al.,* 1999; Pilliteri *et al.,* 2004a, 2004b; Endo *et al.,* 2005; Nishikawa *et al.,* 2007; Tan and Swain, 2007). In *Citrus clementina*, there are three loci encoding FT-like proteins (Samach, 2013). Overexpression of *CiFT, CsLFY, CsAP1*, or *CsSLs* induces an early flowering phenotype in *Arabidopsis* (Kobayashi *et al.,* 1999; Pilliteri *et al.,* 2004b; Tan and Swain, 2007), whereas *Arabidopsis* plants ectopically expressing *CsTFL* shows late-flowering phenotypes (Pilliteri *et al.,* 2004a).
In addition, research by transformation with flowering-related genes has highlighted the long juvenile period in citrus (Peña *et al.,* 2001; Endo *et al.,* 2005). In these experiments, constitutive expression of *Arabidopsis AP1* or *LFY* caused early flowering and fruiting in many of the transgenic citranges [*Citrus sinensis* (L.) Osb. x *Poncirus trifoliate* (L.) Raf.], which initiated flowering in spring, 12-20 months after their transfer to the greenhouse (Peña *et al.,* 2001). The constitutive expression of *CiFT* started to flower as early as 12 weeks after transfer to a greenhouse, whereas wild-type plants usually have a long juvenile period of several years (Endo *et al.,* 2005). The fact that the flowering phenotype resulting from ectopic expression of *CiFT* is an early flowering, researchers have used this method for the rapid evaluation of transgenic citrus flowers and fruits (Endo *et al.,* 2009).

Nishikawa (2013) revised the flowering in Citrus. The genus *Citrus* is different from its close relatives, *Poncirus* and *Fortunella.* Both *Citrus* and *Fortunella* are evergreen, whereas *Poncirus* is deciduous. In satsuma mandarin, floral induction occurs during fall and winter, floral organs are first observable from January through a microscope, and the three different types of shoots in May (Iwasaki, 1959; Inoue, 1990). In respect to the Kumquat (genus *Fortunella*), the bloom is produced during summer and fall, and the floral organ development is observed just before flowering (Abbott, 1935). In trifoliate orange (genus *Poncirus*), evocation happens during early summer (Reuther *et al.,* 1967). The development of flower buds stops during fall and winter, then begins again and the tree different types of shoots are formed in spring. Changes in *CiFT* expression are associated with the seasonal periodicity of flowering (Nishikawa *et*

al., 2007, 2009, 2011; Muñoz-Fambuena *et al.*, 2011, 2012b). In satsuma mandarin (Nishikawa *et al.*, 2009) and 'Moncada' mandarin (Muñoz-Fambuena *et al.*, 2011), *CiFT* expression increases during fall and winter, in which floral induction occurs. This increase results from the expression of *CiFT2* which is also induced during fall and winter in murcott mandarin (*Citrus reticulate* Blanco) (Shalom *et al.*, 2012). In kumquat and trifoliate orange, *CiFT* expression increases during early summer, in which period differentiation of flower organs has been initiated, or just before evocation. The mRNA levels of *CsLFY* remain low during floral induction (winter and fall) in 'Moncada' mandarin (Muñoz-Fambuena *et al.*, 2011) and satsuma mandarin (Nishikawa, 2013); subsequently, levels increase in February and April, respectively, and during this period floral organs develop rapidly. In kumquat and trifoliate orange, mRNA levels of *CsLFY* increase during summer when floral induction and evocation is produced (Nishikawa, 2013). Expression levels of *CsAP1* are moderately high during spring and summer and low during winter (Nishikawa, 2013). In all of these genera, *CsTFL* relative expression was detected at high levels during late spring or early summer, then decreased to undetectable levels. Only *CiFT* showed a close correlation with floral induction in all genera (Nishikawa, 2013).

In *Citrus*, the cool temperature is a decisive factor in floral induction. In some experiments related in satsuma mandarin trees, the plants were exposed to cool temperatures (15°C) and *CiFT* expression increased simultaneously with floral induction (Nishikawa *et al.*, 2007). But when the trees were grown at high temperatures (25°C), vegetative growth was maintained and flowering was not induced (Nishikawa, 2013). At the same time, *CiFT* expression remains at a low level. With these experiments Nishikawa (2013) showed that *CiFT* expression is regulated by temperature. Moreover the same authors showed that in other flowering-related genes, such as *CsLFY*, *CsAP1*, and *CsTFL*, the changes in mRNA levels showed no association with the period of floral induction in trees placed in a growth room at 15°C (Nishikawa, 2013). In fact, *CsLFY* and *CsAP1* relative expression levels increased during the period of evocation of flower buds in satsuma mandarin and sweet orange trees under prolonged 15°C treatment or a subsequent change to high temperature (Nishikawa *et al.*, 2007; Pillitteri *et al.*, 2004b). Only *CiFT* expression showed an increase in response to cool temperature in adult trees and not in juvenile plants (Nishikawa et al., 2007; Nishikawa, 2013). On the other hand, *CsTFL* relative expression was detected at higher levels in young rather than adult plants in trees of satsuma mandarin and sweet orange

exposed to cool temperatures (Nishikawa *et al.,* 2007; Pillitteri *et al.,* 2004a). Nishikawa (2013) hypothesized that the suppression of flowering in young plants might correlate with low *CiFT* expression and high *CsTFL* expression under cool-temperature conditions.

In the last years, changes in gene expression in response to fruit bearing have been reported in 'Murcott' (Shalom *et al.,* 2012), 'Moncada' (Muñoz-Fambuena *et al.,* 2011; 2012b), and satsuma mandarin (Nishikawa *et al.,* 2012). In these genotypes, the levels of *CiFT* expression appear to be high in light-loaded or without fruits "OFF" trees and low in fully-loaded "ON" trees during the floral inductive period. In satsuma mandarin, it was demonstrated that the total *CiFT* expression during fall shows a clear, strong correlation with fruit weight per leaf area (Nishikawa *et al.,* 2012). In addition, the *CiFT* gene expression was associated with the timing of fruit harvest (Nishikawa, 2013). Another experiment done by Nishikawa (2013) consisted in analyzing the *CiFT* relative expression in different branch of the same tree, and *CiFT* expression was high in the branch from which flowers were removed in May and low in the branch from which fruits were harvested in November. This indicated that *CiFT* expression is suppressed by a long fruit-bearing period, and the suppression of *CiFT* expression by fruit bearing is limited to the vicinity to the fruit-bearing portion of the tree. Moreover, the flower number in the following spring showed a positive and high correlation with *CiFT* expression levels during fall and winter in satsuma mandarin (Nishikawa *et al.*, 2012). Taken together, it is considered that excessive fruit amount, a longer period of fruit bearing, and the vicinity to the fruit bearing portion of the tree reduce *CiFT* expression in the stems of vegetative shoots during fall and winter, and correspond with flower number in the following spring (Muñoz-Fambuena *et al.,* 2011, 2012b; Nishikawa, 2013). Given that the flower number and *CiFT* expression show a close correlation (Muñoz-Fambuena *et al.,* 2011; 2012a; 2012b; Nishikawa *et al.,* 2012) and *CiFT* has a function in the promotion of flowering (Endo *et al.,* 2005; Kobayashi *et al.*, 1999), it is believed that fruit bearing suppresses flower number in the following spring via suppression of *CiFT* expression (Muñoz-Fambuena *et al.*, 2011, 2012a, 2012b).

Defoliation of the tree inhibits the flower formation in the following spring (Muñoz-Fambuena *et al.,* 2012b). Moreover, experiments for satsuma mandarin that consisted in removing or decreasing the number of leaves suppressed *CiFT* expression in trees grown under a floral-inductive condition (15°C) (Nishikawa *et al.,* 2013) and at the same time, floral induction was also suppressed in trees from which all leaves had

been previously removed (Muñoz-Fambuena *et al.,* 2012b; Nishiwaka, 2013) or which carried fewer leaves (Niskikawa, 2013). Thus, defoliation suppresses both *CiFT* expression and floral induction. Gibberellin, an inhibitor of flowering in *Citrus*, reduces *CiFT* expression in buds of Orri mandarin (*C. reticulata* Blanco × *C. temple* Hort. ex Y. Tanaka) (Goldberg-Moeller *et al.,* 2013) and leaves of sweet orange (Muñoz-Fambuena *et al.,* 2012a). In sweet orange, paclobutrazol (PBZ), a GA biosynthesis inhibitor, increases flowering by improving *CiFT* relative expression (Muñoz-Fambuena *et al.,* 2012a), which indicates that endogenous GA or applications with GA_3 can inhibit flowering via suppression of *CiFT* expression.

With respect to *FLC* in citrus, a direct inhibitor of flowering, there is little information. *FLC*-like analyzed by Muñoz-Fambuena *et al.,* (2011;2012a; 2012b) did not seem to be relationship with the inhibition of flowering induction in leaves and buds due to the expression initiated after of these period in leaves (Muñoz-Fambuena *et al.,* 2011). On the other hand, other authors analyzed other *FLC*-like, and this showed no differences of expression between treated and untreated buds with GA_3 on OFF trees in the floral induction period (Goldberg-Moeller *et al.,* 2013).

3.2 Quantitative long distance signal regulation of flower induction

The main function of the second level of regulation in perennial trees needs to be a physiological/molecular mechanism that protects/silences particular genes/meristems to be accessible to a floral promoter (Bangerth, 2009). Bangerth (2009) hypothesized that the regulation of flower induction in trees could be regulated far upstream to floral integrator genes, and that they are more similar to general repressor genes like the *FLC* gene or a even epigenetic in nature, which could control the expression of genes and would therefore be particularly appropriate to silence “floral genes”. Because the epigenetic mechanism is able to act over longer distances of time, its action could also explain phenomena like the flower induction inhibition by the fruit, although the fruit had been harvested long ago (Lavee, 1988). Otherwise, the degree of expression of genes of the first level may directly or indirectly be altered by the second level of regulation in a quantitative way, such as DNA methylation (Zemach and Grafi, 2007) or post-transcriptional modifications by particular non-coding RNA species, such as microRNAs (Schmid *et al.,* 2003), which, in some cases, also have epigenetic characteristics.

Although there may be more than just one mechanism that could prevent part of the meristems of the tree from being induced to flower, the non-accessibility of meristems to floral-promoting signals, due to epigenetic control, seems to be an attractive hyphotesis to at least partially explain perenniality (Bangerth, 2009). Examples with *Arabidopsis* demonstrate that this kind of regulation may in fact be one explanation for differences between annual/biennial and perennial plants as well as for effects like shoot bending, girdling, or fruit inhibition on floral induction in trees.

Plant hormones are most prominent amongst long-distance-signals and almost all of them are already extensively used by the plant to interfere with floral induction and other physiological processes such as vegetative growth, dominance phenomena, assimilate partitioning, fruit set and growth, or stress situations (Hoad *et al.,* 1993; Bangerth, 2000). Endogenous as well as applied **gibberellins** have been shown to promote floral induction in some annual/biennial plants and, in addition, to indicate distinct relationships with the level one molecular genetic pathway (Zeevaart, 1976; Blázquez and Weigel, 2000; King and Evans, 2005). Comparisons of endogenous GA levels between rose plants exhibiting seasonal and perpetual flowering habits indicate that GAs might play a role in the duration of flowering season by regulating the return to vegetative development. In seasonal flowering plants, GA_1 and GA_4 levels increase in the shoot apices just after flower initiation, whereas in perpetual flowering plants GA levels are low throughout the year (Roberts *et al.*, 1999). King and Evans (2005) demonstrated in detailed experiments with their "model plant" *Lolium temulentum* that GA_5 is a "florigenic" signal, possibly intermediate between long-day perception and *FT* expression (King and Evans, 2005). In some species of gymnosperm trees, applied low polar GAs are able to induce precocious and profilic floral induction (Pharis and King, 1985). However, in most angiosperm trees, GAs have the opposite effect, that is, they considerably inhibit floral induction applied exogenously (Guardiola *et al.*, 1982; Tromp, 1982; Oliveira and Browning, 1993; Prang *et al.*, 1997; Mutasa-Göttgens and Hedden, 2009), as evidenced in the assays appliying GA_3 (Ayalon and Monselise, 1960). When dealing with endogenous GAs, however, the situation becomes more complex (Oliveira and Browing, 1993; Bernier and Périlleux, 2005). The problems arise when considering GAs as directed long-distance signal to inhibit floral induction in trees. A better understanding of GAs as floral inhibiting long-distance-signals began with the findings of Chan and Cain (1967) who demonstrated that seeded but not seedless apple fruit inhibits floral induction for the next season. This finding was

understandable taking into account the high concentration of GAs in the seeds of apple and other fruit (Hedden *et al.*, 1993). The same result was obtained in pears (Griggs *et al.*, 1970) and citrus (Monselise and Goldschmidt, 1982).

In experiments with apple, inhibition of floral induction by fruit/seeds was generally mediated mainly to nearby meristems. The effect of GAs on floral induction is confused by a number of tree-specific interfering factors, like vegetative growth in apple (Neilsen and Dennis, 1999) and citrus (Krawjewski and Rabe, 1995), the number of leaves in pears (Huet, 1972). Exogenous treatments usually reduced floral induction in a number of tree species (Goldschmidt and Monselise, 1970). However, the results were often variable and the concentrations needed to obtain significant effects were generally high and not of normal physiological concentrations. In addition, there are considerable structural differences in GAs which greatly affect their inhibiting action (Tromp, 1982; Oliveira and Browing, 1993). The most significant effect in GA-efficiency in this respect is the way GAs are applied to the plant. Goldschmidt and Monselise (1970) treated single buds of orange shoots with GA_3 and obtained a 75% reduction in the average number of flowers/shoot, with higher concentrations causing a complete inhibition of floral induction. Bertling and Bangerth (1995) injected either GA_3 or $GA_{4/7}$ into the trunk of numerous fruit tree species and cvs. and obtained a considerable reduction of the floral induction. This method confirmed that when spraying GAs an obviously high proportion of the hormone is conjugated and/or metabolized or does not reach the intended target, which could explain the low or variable efficiency of spraying. When applications are done in a more appropriate physiological way, GAs are able to keep many above-ground buds in a vegetative non-accessible state even if applied at physiological concentrations. The way in which this non-accessibility is maintained is presently not known but may involve chromatin as well as rejuvenation effects.

A “directed” transport of GAs from a defined source such as fruit/seed or shoot tips to a specific target has not yet been clearly demonstrated. In fact, it would be difficult to assume transport from a fruit, a strong sink, to the apical bud of an apple bourse shoot, which is a weak sink. Nonetheless, Prang *et al.* (1997) reported an export of GAs out of apple fruit as well as shoot tips with a peak occurring at the time presumed floral induction does occur, but there was little correlation between the regular and the alternate-bearing cvs. Due to the techniques applied, the destination of these GA-signals could not be determined. Using the same cvs. in the same years,

Stephan *et al.* (2001) used LC-ESI-MS and internal standards to investigate the export and metabolism of GAs out of these fruits. In fruit exudates of the regular bearing cv.,they found six different GAs with GA_4 prevailing over GA_3. In the biennial bearing cv. 'Elstar', however, GA_3 was the predominant signal. When radioactive labelled GAs were injected into the core of the fruit and their export and metabolism in the pedicel and spur followed, the same authors found a high proportion of GA-metabolites or glycosylated conjugated, both of which are biologically inactive (Stephan *et al.*, 2001). No intact GA-molecules could be found in the apical bud of the shoot which is usually induced to flower. Seeds are not the only source of GAs relevant for floral induction in trees. Shoot tips are also rich in bioactive GAs, particularly GA_1, and these may be involved in the floral inhibition of lateral buds of long shoots, for example, during strong vegetative growth (Forshey and Elving, 1989). Boss *et al.,* (2004) showed that a mutant of *Vitis vinifera*, deficient in its response to GAs, only produced inflorescences and no tendrils, whereas the wild type, which showed full GA-sensitivity produced only tendrils.

In citrus, there are two key moments in which the exogenous application of GAs has the maximum efficiency on the inhibition: during translocation of the flowering signal of leaves to buds (the end of November to early December) and the beginning of the morphologic differentiation of flowers (Monselise and Halevy, 1964; Guardiola *et al.*, 1982). As the effect of the exogenous application of GAs is similar to that produced by the fruit, Goldschmidt and Monselise (1970) suggested that the mechanism by the fruit inhibit the flowering is through the synthesis of GAs. In fact, in the Satsuma mandarin, the GAs concentration (GA_1 / $_3$) is higher in the leaves of ON shoots in October (Koshita *et al.,* 1999). But its concentration decreased to a similar level in both types of shoots in December, coinciding with the flowering induction in this genotype (December-January). Therefore, their relationship with the inhibition of floral induction cannot be determined.

Bangerth (2009) suggested that **polar IAA transport,** a long-distance-signal, could act as a secondary messenger to GAs. IAA is the only plant hormone with a strictly polar, highly-regulated transport pathway (Muday and DeLong, 2001), and IAA signals are therefore independent of sink or transpiration driven transport. 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. There are indications which seem

to support a role for IAA transport in floral induction and its function as second messenger. The application of the gibberellins GA_3, GA_4 and GA_7 to apple trees considerably stimulated IAA-export of fruit as well as of shoot tips (Callejas and Bangerth, 1997), which would be in line with a second messenger role for IAA. A particular class of IAA-transport inhibitors (morphactin and TIBA) stimulates floral induction in a number of fruit trees (Luckwill, 1969; Ito *et al.*, 2001; Bangerth, 2009) and a number of horticultural procedures are used to increase floral induction, like bending, girdling, shoot tip removal or damage, these considerably reducing polar IAA transport (Blaikie *et al.*, 2004). Lastly, application of an auxin to a decapitated shoot tip is also reported to inhibit floral induction (Tamim, 1996).

In contrast to GAs and to polar IAA transport, it has been shown that the application of **cytokinins (CKs)** promotes floral induction in monocarpic and polycarpic plants (Ramirez and Hoad, 1981; Bernier and Périlleux, 2005). Applications of low concentration of CKs had no effect on floral induction (Bernier and Périlleux, 2005) while transgenic CK-deficient *Arabidopsis* plants never flowered (Werner *et al.*, 2003), hence the essential role of CKs in floral induction. At medium CK concentrations, floral induction occurs, but high concentrations promote only vegetative development; these positive effects are seen in *Sinapis alba* (Bernier and Périlleux, 2005), *Vitis vinifera* (Srinivasan and Mullins, 1981) and *Malus domestica* (Bangerth, 2009). Other experiments, (Stern *et al.*, 2003) focusing on the effect of water stress to increase the concentration of xylem CKs, found a concomitant quantitative increase in floral induction. Application of other, non-CK compounds, like maleic-hydrazide and triidobenzoic acid (TIBA), have also significantly increased floral induction of apple, pear, olive and mango trees (Luckwill, 1969; Ben-Tal and Lavee, 1985; Ito *et al.*, 2001; Blaikie *et al.*, 2004). Ito *et al.* (2001) reported considerable increases in the endogenous CK concentrations of lateral and terminal buds on trees treated with these substances. Both maleic-hydrazide and TIBA are potential inhibitors of the IAA polar transport and/or metabolism, the CK increase may be the result of a reduced IAA concentration either in the buds themselves or in the roots (Bangerth, 2000) so that CKs may be the cause for the increased floral induction rather than the IAA transport.

Another important hormone is ABA, which is transported acropetally as basipetally (Davies, 2010). In assays with citrus Shalom *et al.* (2014) identified a transport of ABA from fruit to buds. The content of ABA was higher in ON. However, the *NCED3* genes, which determine synthesis of ABA, had presented a higher

expression in OFF buds. Therefore researchers suggest that the content of ABA in ON buds is produced in other part of the tree, particularly, the fruit (Shalom *et al.*, 2014). In early studies relating ABA with the inhibition of flowering, Goldschmidt and Golomb (1982) observed a higher ABA concentration and its isomer t-ABA in leaves, stems and buds of ON trees in December, suggesting their relationship with dormancy and the inhibition of flowering. On the other hand, exogenous ABA treatment to buds of *Citrus unshiu* reduced sprouting and flowering of these buds (García-Luis *et al.,* 1986). Nevertheless, in stress conditions flowering is promoted (Koshita and Takahara, 2004), and this is coincident with an increase in ABA content in leaves (Gómez-Cadenas *et al.*, 2000; Koshita and Takahara, 2004) and an increase in *CiFT* expression (Chica and Albrigo, 2013). The relationship of ABA with flowering in citrus has yet to be clarified.

Floral induction in annual/biennial plants has been linked to the induction and the release of dormancy (Horvath *et al.,* 2005). It is generally agreed that dormancy of both bud and cambial meristems is largely controlled by plant hormones, mostly ABA, cytokinins and gibberellins (Powell, 1987; Lombard *et al.*, 2006; Nieminen *et al.*, 2008) and their interaction with DNA methylation/demethylation and histone/chromatin modifications.

4. Hypothesis and objectives

4.1 Hypothesis

In mature citrus trees, flowering is regulated by exogenous signals (i.e., low/high temperature, water shortage/water supply) that control flowering time, and by endogenous signals that control flower intensity. According to Martínez-Fuentes *et al.* (2004), three levels of flowering can be established depending in the promoter/inhibitor (P/I) ratio. The first is one of high flower intensity, defined by a high P/I ratio in which P reaches a high level and exogenous inhibitors cannot counteract the promoter. In contrast, a low flowering level is due to a low P/I ratio in which the inhibitor prevails over the promoter and the application of the latter cannot counteract the inhibitor. Only at a nearly balanced P/I ratio can flowering be exogenously inhibited by applying inhibitors (i.e. GA_3), or promoted, by applying promoters (PBZ) (**Fig. 5**). The ratio of endogenous promoters to inhibitors is considered as responsible for flowering; however, there is evidence suggesting that the inhibitors and their metabolism are the sole factors controlling flowering in *Citrus.* According to Agustí (1980), all the meristems have the information and ability to flower unless a negative factor hampers the process. The main endogenous factor controlling flower intensity is the fruit. Bangerth (2009) proposed the existence of long-distance signals (hormonal in nature) needed to silent the transcription of genes involved in the floral process through epigenetic mechanisms.

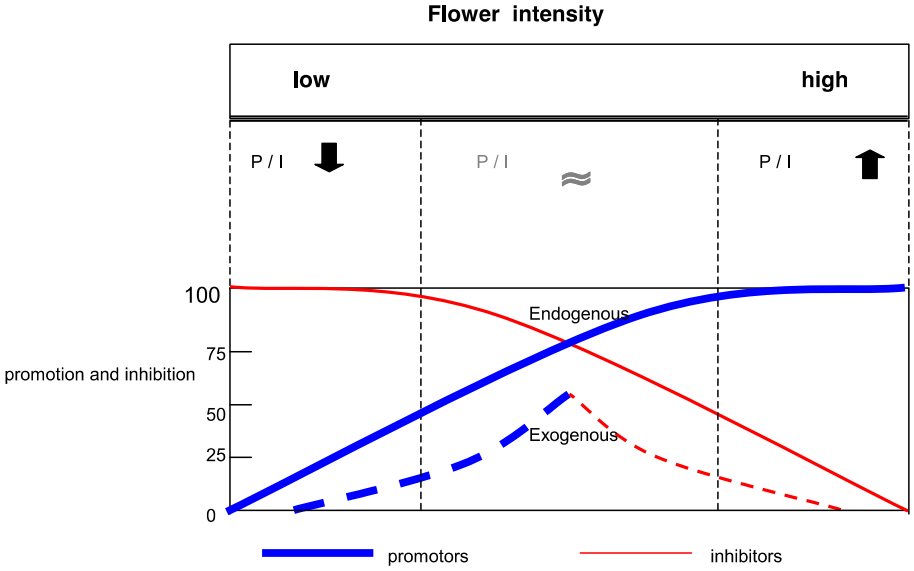


Figure 5. Schematic diagram of the ratio of endogenous flowering inhibitors and promoters and exogenous flowering inhibitors and promoters and their effects on controlling flowering in *Citrus* (From Martínez-Fuentes *et al.*, 2004).

In this PhD. thesis, the following hypothesis was tested:

Fruit inhibits flowering when ripening begins by exporting hormones that induce epigenetic upregulation of flowering inhibitor genes in the leaves, and interfering in flower bud differentiation.

4.2 Objectives

Therefore, the following objectives were established:

1. To correlate the endogenous content of GAs and ABA in the fruit, leaves and buds, and their biosynthesis, with the expression of flower induction and flower differentiation genes.

2. To identify the effect of bud isolation from the fruit (girdling and *in vitro* culture) on sprouting and flowering.

3. To determine the influence of the fruit in the proteome of buds and leaves.

4. To characterize the effect of the fruit in DNA methylation of gene promoters and inhibitors of flower induction.

Materials and method

1. Plant material and experimental design

1.1 Previous experiments

1.1.1 Characterization of *Flowering Locus C*

The material used as experimental model was *Arabidopsis thaliana*, concretely the seeds of the ecotypes Columbia 0 (Col-0) and Landsberg *erecta* (Ler).

The culture was done in the greenhouse, in culture chambers and in fitotron under light conditions (16 hours of light and 8 hours of darkness), relative humidity (50-80%) and temperature (21-23ºC) controlled. As substrate, we used a mix of peat moss:perlite:vermiculite (2:1:1) arranged in each container of polyethylene; each container was irrigated with a mix of nutritive solutions (Hoagland, 1920).

1.1.2 Determination of *Flowering Locus T* responsible for floral induction

This study was conducted comparing 'Cleopatra' mandarin (*Citrus reshni* Hort. Ex Tan.) juvenile plants and adult 'Moncada' mandarin (Clementine Oroval [*Citrus clementine* Hort. ex Tan.] x 'Kara' mandarin [*C. unshiu* Marc. x *C. nobilis* Lou.]) trees, grafted onto Carrizo citrange (*C. sinensis* L. Osbeck x *Poncirus trifoliata* L.Raf.) rootstock, planted 5 m x 5 m apart in a loamy-clay soil, with drip irrigation. The experimental field was located in the IVIA Research Station (Moncada, Spain). Three trees were selected for homogeneity in diameter, canopy height, size and shape and a randomized complete-block design was employed. Three plants of 'Cleopatra' mandarin were selected for each cultivar in July (2012). 30 leaves per cultivar were sampled in September, November and February for RNA extraction and RT-PCR analysis for the study of *Flowering Locus T* (*FTs)* expression. Samples were immediately ground and stored at -80ºC for further analyze.

1.2 Section 1

1.2.1 Experiment I

In this experiment the 'Moncada' mandarin from IVIA was also used. From early September (2013) to the end of February (2014), 30 ON shoots and OFF shoots were collected. In the laboratory, these shoots were separated into developed mature adult leaves, buds and exocarp from ON (fully loaded) and OFF trees (without fruits). Previously, the fruits were measured for their diameter, color of exocarp, chlorophyll and carotenoid contents. At the same time in September a sample of fifteen OFF shoots was taken and the leaves of different sprouting were separated (spring+summer or fall). From these, half of the leaves and buds were collected for RNA extractions and RT-PCR analysis for the study of *Flowering Locus T 2* (*CiFT2), Flowering Locus C (CcFLC), Gibberellin 20 oxidase 1 (GA20ox1)* and *Gibberellin 3 oxidase 1 (GA3ox1)* expression and the other half were used to sample the exocarp of fruits for GA and IAA concentration analysis by UPLC-MS/MS. All samples were immediately ground and stored at -80ºC until the time for analysis.

1.2.2 Experiment II

Experiments were carried out during 4 consecutive years (2012-2015) using adult trees (10–15 years old) of 'Salustiana' and 'Navelina' sweet oranges (*Citrus sinensis* L. Osb), 'Hernandina' Clementine (*Citrus clementina* Hort. ex Tan.) and the two hybrids 'Afourer' (*Citrus reticulata* × *C. sinensis* L. Osb) and 'Moncada' mandarin. Oranges 'Salustiana' and 'Navelina' were planted 5 m × 6 m apart and grown in a loamy–clay soil and sandy–loamy soil, in orchards located in Valencia (39.28ºN–0.22ºW, 30 m altitude) and Huelva (37.22ºN–6.58ºW, 26 m altitude), Spain, respectively. The 'Hernandina' Clementine and 'Moncada' mandarin trees were planted 5 m × 5 m apart in a loamy–clay soil in Valencia. The 'Afourer' mandarin trees were planted 4 m × 6.5 m apart in a sandy–loamy soil in Huelva. All the orchards had drip irrigation, and fertilization, pest management and pruning were in accordance with normal commercial practice.

Paclobutrazol (PBZ) was applied during either the floral bud inductive period (November 20–25) or the floral bud differentiation (February 20–24). PBZ was applied either to the soil, directly to the drip-line zone (1–10 g tree^{-1}), or sprayed on the canopy

by handgun (6 l tree^{-1}, 2500 mg l^{-1}, i.e. 15 g tree^{-1}). Early in the rest period (October 10–15) treatments were also carried out for comparison.

Gibberellic acid (GA_3), at a concentration of 50 mg l^{-1}, was sprayed on the canopy in the floral bud inductive period for comparison. Six to ten ON- and OFF-trees were randomly selected for each cultivar, orchard and treatment. Untreated ON- and OFF-trees were used as controls. Four homogeneous branches per tree containing spring, summer and fall flushes, with some 400 nodes per branch, and randomly distributed around the canopy, were selected on the treatment date for flowering evaluation in the following spring.

In a separate experiment, the response of buds to PBZ was studied by placing a 10 µl drop of a 2500 mg l^{-1} aqueous solution directly on buds of ON- and OFF-trees of 'Hernandina' mandarin. This treatment was carried out during the floral bud inductive period. One hundred buds were randomly selected from 10 ON-trees and 10 OFF-trees for treatment, and another hundred served as control.

In addition, another experiment was done to analyze the inhibitory effect of fruit on flowering. Only trees bearing moderate fruit loads (*80 kg tree^{-1}) were selected of 'Salustiana' sweet orange. In early December, 40 mg L^{-1} of GA_3 and 2,000 mg L^{-1} of PBZ were sprayed onto the entire tree with a hand-gun sprayer. Untreated trees served as control. From the treatment date (11 December) to the onset of bud sprouting (late February), 30 fully developed, autumn flush (that is, nonbearing shoots), mature adult leaves per tree from control and another 30 from GA_3- and 30 from PBZ-treated trees were randomly collected for RNA extraction. Samples were ground and stored at –80ºC for RNA extraction and RT-PCR analysis for the study of *CiFT, FLC*-like, *GA20ox1* and *LEAFY (CsLFY)*. Six trees were used for the extractions. All of these experiments were evaluated the yield, the sprouting and the flowering.

1.2.3 Experiment III

This experiment was done in 'Afourer' mandarin trees situated in Pedralba (Valencia) with normal drip irrigation, fertilization and culture. 100 leafy single flowered shoots (ON) were selected from 5-6 nodes long and 70 of vegetative shoots (OFF) from 10 trees. In half of the ON shoots girdling was done for the peduncle at the end of August (25 August, 2014). Girdling was performed by removing a 2-mm ring of bark from the peduncle 1.0 cm from the calyx. Fruits from ON shoots that were not girdled served as

controls. At the end of summer and spring the sprouting and the flowering in 30 shoots per treatment were evaluated. In these periods the characteristics of the fruit were evaluated: weight (g), diameter (mm) and the color the peel and the pulp, the total soluble solids (TSS) and the length of the shoots formed. For the endogenous hormonal analysis, 20 shoots were collected for each treatment before the onset of sprouting (11 days after girdling, September 5).In the laboratory OFF shoots were separated in different nodes (node 1 nearer of apex than node 5) and the same ON shoots. In ON shoots the peel of the pulp was also separated. In these samples GA_1, GA_4, IAA, Tz, ABA and JA concentration by UPLC-MS/MS were analyzed. All samples were immediately ground and stored at -80ºC until analyzed. Previously to analyze the samples were lyophilized.

1.2.4 Experiment IV

This study was conducted with 10-year-old field-grown trees of 'Mandarino tardivo di Ciaculli' mandarin (*Citrus reticulata* Blanco), grafted onto *Citrus aurantium* L. This experiment was done in an experimental field located at the Università degli Studi di Palermo (Palermo, Italy) with normal drip irrigation, fertilization and culture.

In this experiment 20 vegetative shoots (OFF) and 20 leafy single flowered shoots were collected in the induction period (November, 2013) and in February. The microshoots from these shoots were selected to cultivate *in vitro*. The microshoots were 1.0 cm long and each microshoot had a bud. The MS medium (Murashige and Skoog, 1962) was used with and without zeatin. The percentage of sprouting was evaluated every 7 days for 60 days. In the induction period (November 13) and in February the characteristics of the fruit were evaluated: weight (g), diameter (mm), the color the exocarp and number of seeds. Moreover, in spring, sprouting and flowering were evaluated in 30 shoots per treatment.

1.3 Section 2

'Moncada' mandarin trees located in the IVIA orchards were used in these experiments. Spring flush leaves and buds from 8 month old trees were used as biological material and were randomly collected from 12 trees (6 on-crop and 6 off-crop) in autumn, which is when the fruit affects floral induction (November 2010). All leaves and buds

were harvested the same day at 11:00 a.m. and were immediately frozen and stored at -80ºC. These samples were collected by protein extraction and subsequent analysis.

In another experiment, 30 ON shoots and 30 OFF shoots were collected from early September to the end of January. In the laboratory, these shoots were separated into developed mature adult leaves, buds and exocarp from ON (fully loaded) and OFF trees (without fruits). Previously, the fruits were measured for the color of exocarp and the carotenoid content. The samples were collected for ABA and JA concentration analysis by UPLC-MS/MS. All samples were immediately ground and stored at -80ºC until analysis.

1.4 Section 3

In these experiments 'Moncada' mandarin trees located in the IVIA orchards and 'Afourer' mandarin trees located in Pedralba were used.

In all experiments flowering in spring was evaluated. In the first experiment, from early January (Year 1, 2014) to the end of February of the next year (Year 2, 2015), ON and OFF buds (January Year 1) and 10 ON leaves and 10 OFF leaves were collected for each sample (Year 2). In the second experiment a selection was made from 3 branches per tree, OFF, ON and defruiting (defruited in August 25, 2014) in 3 different trees. 10 ON, 10 OFF and 10 defruiting leaves were collected at the induction period (November 25, 2014). In the third experiment 100 ON shoots taken from 3 trees were selected. Half of the shoots were used as control. The other half of these leafy single flowered shoots were sprayed with a hand-gun sprayer containing 5-Azacytidine, 350 µM of solution. After the treatment, the samples were taken at 8, 24 and 48 hours. The leaves and buds of these samples were collected for RNA extractions and RT-PCR analysis for the study of *CiFT2, FLC, SHORT VEGETATIVE PHASE (SVP), TEMPRANILLO 1 (TEM1), EARLY FLOWER 8 (ELF8), TRITHORAX 1,2 (12ATX), TRITHORAX 7 (7ATX), PROTEIN ARGININE METHYLTRANFERASE 5 (SKB1)* and *TRITHORAX 5 (5ATX)* expression. Moreover, samples from OFF and ON leaves in May and November were used for DNA extraction and DNA methylation analysis of *FLC* in May and *Flowering Locus T 1* (*CiFT1), CiFT2, FLC, SVP* and *TEM1* in November. All samples were immediately ground and stored at -80ºC until analysis.

2. Method

2.1 Yield, sprouting and flowering evaluation

Sprouted buds, developing shoots and flowers as well as leaves per shoot were counted and evaluated following to Guardiola *et al.* (1982). Unsprouted nodes were also recorded. The total number of flowers per branch was calculated using the number of flowers per shoot and the number of shoots developed per branch. The results are expressed in flowers per 100 nodes to compensate for the differences in the size of the branches selected for counting. Fruits were harvested at the appropriate commercial size and color standards, and yield (kg tree^{-1}) was determined by weighing and counting all fruits harvested per tree.

2.2 Fruit characteristics

Fruits were previously evaluated for weight (g), diameter (mm) with a caliper (Mitutoyo, Japan), the color of the exocarp and the endocarp by a colorimeter and by Minolta Chromameter CR-300 (Minolta, Japan) and the total soluble solids with a digital refractometer (Atago, Japan) and number of seeds.

Three measurements per fruit in the equatorial zone of the fruit were taken from October until January or March in Moncada (Valencia), Palermo (Sicily) and Pedralba (Valencia). The results are given as a, b and L Hunter coordinates. a Hunter is indicative of peel transition from green to orange in *Citrus* and because zero coincides with the onset of fruit color break. Color readings of a denote green or red when negative or positive, respectively. b Hunter denotes blue or yellow when negative or positive, respectively, and L Hunter denotes black or white when is 0 or 100, respectively.

In addition, pigment analysis chlorophylls and carotenoids were extracted from frozen exocarp as described in Rodrigo *et al.* (2003). Chlorophyll a, b and total (a + b) were determined by measuring absorbance at 644 and 662 nm. Calculations were performed following Smith and Benitez's (1955) equations. After chlorophyll measurements, the pigment ethereal solution was dried and saponified with 10% methanolic KOH solution. Carotenoids were subsequently re-extracted with diethyl ether until the hypophase was colorless. Total carotenoid content in the ethereal extract was calculated measuring absorbance at 450 nm and using the extinction coefficient of β-

carotene (ϵ = 2500; Davies, 1976). Three independent extractions were performed per sample.

2.3 Culture

2.3.1 Biological materials

2.3.1.1 Seed preparation and sterilization of *Arabidopsis thaliana*

For the sterilization of the seeds, two protocols were followed:

a) Sterilization by chlorine atmosphere. Seeds were placed in an open microcentrifuge tube and after these seeds were placed in a desiccator which contained a beaker with 100 ml bleach. Next, 3 ml of 37% hydrochloric acid was added to the bleach and the desiccator was immediately closed. It was left standing between 1-4 hours. This sterilization was performed in a fume cupboard.
b) Sterilization with ethanol. In a microcentrifuge tube containing the seeds 200 µl of 70% ethanol + 0.05% Triton X-100 was added. The tube was gently shaken for 3 minutes to wash the seeds. The liquid was removed, and after that 200 µl of 96% ethanol was added and the tube was shaken for 1 minute. Next, the seeds were placed on sterile filter paper moistened with 96% ethanol and allowed to dry.

For the sowing of the seeds two protocols were followed:

a) Direct sowing in substrate. Pre-sterilization is not necessary. Seeds were immersed in culture tube with 0.05% agarose solution. The tube was shaken to distribute homogenously. The seeds were stratified after 3 days in darkness at 4ºC. Finally, the suspension was distributed in polyethylene containers with moistened substrate.
b) Sowing in plates of MS. After sterilizing the seeds, they were distributed homogeneously in Petri plates with MS medium. The plates were closed with porous tape and they were stratified by treatment over 3 days in darkness at 4ºC. After 3 days, the plates were taken out and placed in a culture chamber.

To transfer the seedlings that were grown *in vitro* to soil, we used curved forceps. These seedlings were distributed in polyethylene containers with moistened substrate.

2.3.1.2 Transformation of *Arabidopsis thaliana* with *Agrobacterium tumefaciens* by *FLC* characterization

The method used for the transformation was immersion "floral dip". The modified protocol of Clough and Bent was followed (Clough and Bent, 1998). First of all, the plants were cultured until flowering (4-5 weeks approximately) in individual containers with substrate. Secondly, the *Agrobacterium tumefaciens* C58 suspension was prepared with the correspondent construction. Next, 1 liter of the saturate culture was used for the transformation and this was centrifuged 10 minutes at 4000 rpm. After this, the pellet was resuspended in infiltration medium: 5% saccharose + 0.02% Silwett detergent. Finally, plants were immersed for 1 minute in *Agrobacterium tumefaciens* C58 suspension. Then, the plants were placed on paper to remove excess of suspension. They were left in darkness for 24 hours. Subsequently, the plants were cultured under normal conditions of relative humidity, light and temperature until the seeds were collected.

2.3.1.3 Microshoot sterilization and culture of 'Mandarino Tardivo di Ciaculli'

Sterilization and culture:

First of all, the leaves and fruits were removed. Then, the shoots were cut in microshoots of 1 cm with one bud. After that, the material was washed with water and 10 ml detergent at the same time that the each shoot was cleaned with a toothbrush. Next, the material was placed in a vacuum pump for 20 minutes. Then microshoots were sumerged in fungicide 2g l^{-1} for 10 minutes (also in vacuum pump). Subsequently, the material was washed with water and they were placed in the laminar flow hood..

Under the laminar flow hood, the microshoots were immersed firstly for 5 min in 70% (v/v) ethyl alcohol (vacuum), then in 35% commercial bleach solution with few drops of Tween 20, for 15 min. (vacuum). Finally, they were rinsed three times with sterile distilled water and immerged in left standing for 30 minutes with water and antibiotic (Cefotaxime 0.2 mg ml^{-1}). Ten microshoots were put per each Petri dish containing 25 mL of medium before the incubation at 27±1 °C, under cool white fluorescent lamps (Philips TLM 30W/84) with a Photosynthetic Photon Flux Density of 35 μmol m^{-2} s^{-1} and a 16 h light photoperiod.

The culture were observed for 2 months, every week.

Medium composition:

For the culture, MS basal salt medium was prepared (Murashige and Skoog, 1962). The vitamin supplement consisted of 1 mg l^{-1} thiamine–HCl, 1 mg l^{-1} pyridoxine–HCl, 1 mg l^{-1} nicotinic acid, 2 mg l^{-1} glycine, 5 mg l^{-1} calcium pentathenate and 100 mg l^{-1}*myo*-inositol. The medium also contained 30 g l^{-1}sucrose and 8 g l^{-1}agar (agar-agar/gum agar) (Sigma Chemical, St. Louis, MO). The medium was balanced to pH 5.85 with 1N KOH, and autoclaved at 121 °C and $1x10^5$ Pa (1.1 kg/cm^2) for 21 min. Two media were prepared. The first MS medium without growth regulator and the second MS medium with zeatin (1 mg ml^{-1}). In addition, 1.5 ml PPM l^{-1} was added to each medium.

2.3.2 Bacterial strain

Two types of bacterial strain were used:

1. DH5α strain of *Escherichia coli* by the production and maintenance of plasmids.
2. C58 strain of *Agrobacterium tumefaciens* by transformation of *Arabidopsis thaliana.*

Bacterial cells were grown in LB medium (yeast extract 0.5%, triptone 1% and NaCl 1%) liquid and solid (with agarose 1.5%) supplemented with the corresponding antibiotics to the resistance genes that the plasmids had. *E.coli* was cultivated for 1 day at 37ºC while *A.tumefaciens* was cultivated for 2 days at 28ºC, both under agitation. When the plasmid contained genes of selection by color, 40 µl of X-Gal (40 mg ml^{-1}) was added to the medium.

The constructions produced were kept in a freezer at -80ºC in glycerin form. Next, 700 µl of a saturate liquid culture of the bacterial strain was mixed with 300 µl of 50% glycerol. Finally, it was frozen in liquid nitrogen and stored until use.

2.3.2.1 Transformation of *Escherichia coli*

Two methods of transformation were used:

a) Transformation by heat shock method. DNA sample was dialyzed (50 µg when is a ligation) before that it was introduced into the bacteria. The drop was situated on a nitrocellulose filter of 0.025 µm in form of disc of Millipore. It was placed in Petri dish with miliQ water for 10 minutes. After that, DNA was collected and

mixed with chemically competent cells that were in ice at 4ºC. This mixture was left to stand for 30 minutes in ice. After this incubation in ice, a mixture of chemically competent bacteria and DNA was placed at 42°C for 1 minute (heat shock) and then placed back in ice. 500 µl of SOC media was added and the transformed cells were incubated at 37°C for 1 hour with agitation. Finally, the mix was poured into the Petri dish with the LB and the antibiotics.

b) Transformation by electroporation. DNA sample was dialyzed and mixed with electro competent cells that were in ice at 4ºC. The mixture was introduced in electroporation cuvettes, dry and cool. These cells were subjected to an electric pulse of 1500V for 5 ms. 250 µl of SOC media was added in the cuvette and this mix was transferred to a microcentrifuge tube. After that, the tube was incubated at 37°C for 1 hour with agitation. Finally, the mixture was poured into the Petri dish with the LB and the antibiotics.

2.3.2.2 Transformation of *Agrobacterium tumefaciens*

For this, transformation by electroporation was used. The DNA sample was dialyzed and mixed with electro competent cells that were in ice at 4ºC. The mixture was introduced in electroporation cuvettes, dry and cool. These cells were subjected to an electric pulse of 1440V for 5 ms. 1 ml of SOC media was added in the cuvette and this mixture was transferred to a microcentrifuge tube. After that, the tube was incubated at 28°C for 2-3 hours with agitation. Finally, the mixture was poured into the Petri dish with the LB and the antibiotics.

2.4 Molecular biology techniques

2.4.1 Extractions

2.4.1.1 Protein

Frozen Moncada leaves and buds from each tree were separately pulverized in liquid nitrogen with 0.05% PVP (twelve samples). Then, samples were homogenized in an extraction buffer (50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 0.2% β-mercaptoethanol) using a pestle and mortar. Homogenates were centrifuged for 20 min at 20,000 g. The

supernatants were mixed with an equal amount of cold 20% TCA. The mixtures were incubated for 1 h at 4ºC and centrifuged at 20,000 g for 15 min at 4ºC. The protein pellets were washed three times with acetone. After the last centrifugation at 20,000 g for 15 min at 4ºC, pellets were re-suspended in a lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS). Protein aliquots analyzed by 2D electrophoresis were stripped of non-protein contaminants using a 2D Clean-Up Kit following the manufacturer's instructions (GE Healthcare). The cleaned protein was re-solubilised in a lysis buffer the conventional 2D analysis or in a Tris-buffered solution (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-HCl pH 8.5) for 2D DIGE analysis. Protein concentration was determined with the Bio-Rad protein assay using bovine serum albumin (BSA) as standard.

2.4.1.2 RNA and genomic DNA

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 Citrus actin primers (**Supp. Table 1**). No amplified products were detected, which confirmed the purity of the RNA extracts. RNA extracted was stored in a freezer at -80ºC until use. Genomic DNA was extracted from frozen tissues. DNA extracted was frozen at -20ºC until use.

2.4.1.3 Extraction of plasmid DNA

For the plasmid DNA extraction of *Escherichia coli* 5 ml of the liquid culture was used and the system used for this was "Plasmid Mini Kit I (E.Z.N.A).

2.4.2 Protein analysis and measurements

2.4.2.1 Fluorescent labelling

Protein samples were labelled using the CyDyes DIGE fluors (Cy2, Cy3 and Cy5) according to the manufacturer's instructions (GE, Healthcare). Three different protein

samples (internal standard, on and off samples) were labelled individually with the three dyes. The internal standard was created by pooling an aliquot of all biological samples analyzed in the experiment and it was always labelled with Cy2. Six biological replicates were analyzed in this experiment; thus, twelve biological samples were used to make the internal standard. The sample of each on-crop tree never was mixed with samples of other ON-crop trees (the same for OFF-crop samples). The ON sample and the OFF sample were labelled with Cy3 or Cy5, alternatively, depending on the biological replicate, thus avoiding the label effect. Equal amounts (50 mg) of ON (Cy3, for example), OFF (Cy5) and internal standard (Cy2) samples of the same biological replicate were pooled. A lysis buffer was added to make the volume up to 40 mL. Then, the sample was mixed with 40 mL isoelectrofocusing (IEF) rehydration buffer (8 M urea, 4% CHAPS, 0.005% bromophenol blue) containing 65 mM DTT and 1% IPG buffer pH 3-11 and loaded into the gel (one gel for each biological replicate).

2.4.2.2 2D electrophoresis

For 2D analysis, strips of 24 cm in length with immobilized pH gradient of 3-11 were hydrated overnight at room temperature with 450 mL IEF rehydration buffer, containing the reagents Destreak and Pharmalyte pH 3-10, according to the manufacturer's instructions (GE, Healthcare). CyDyes labelled samples (150 mg of protein) were loaded in hydrated strips. IEF was performed on an IPGphor unit (GE Healthcare) at 20ºC and a maximum current setting of 50 mA per strip, using the following settings: 300 V for 1 h, an increasing voltage gradient to 1000 V over 6 h, an increasing voltage gradient to 8000 V over 3 h, before finally holding at 8000 V for a total of 32,000 V h. After IEF, each strip was equilibrated separately for 15 min in 10 mL equilibration solution I (0.05MTris-HCl buffer, pH 8.8 containing 6 M urea, 30% glycerol, 2% SDS, 200 mg DTT per 10 mL buffer) followed by equilibration solution II (substituting DTT for 250 mg iodoacetamide per 10 mL buffer and adding 0.01% bromophenol blue) before being applied directly to the second dimension 12.5% SDS-PAGE gels. Six gels were run simultaneously at 20ºC, applying 2 W/gel for 30 min and 20 W/gel for the remaining 5-6 h, using an Ettan DALTsix unit (GE, Healthcare). A running buffer of 25 mM Tris pH 8.3, 192 mM glycine and 0.2% SDS was used. Each gel showed the differential protein expression between an ON sample (from a single ON-crop tree) and an OFF sample (from a single OFF-crop tree).

2.4.2.3 Gel imaging and data analysis

After SDS-PAGE, CyDye-labelled proteins were visualized by scanning using a Typhoon Trio scanner (GE Healthcare) with the relevant wavelengths for each CyDye. Cy2 images were scanned using a blue laser (488 nm) and an emission filter of 520 nm band pass (BP) 40. Cy3 images were scanned using a green laser (532 nm) and a 580 nm BP 30 emission filter. Cy5 images were scanned using a red laser (633 nm) and a 670 nm BP 30 emission filter. All gels were scanned at 200 mm (pixel size) resolution. The photomultiplier tube (PMT) was set between 500 and 600 V using normal sensitivity. The scanned gels were then directly transferred to the ImageQuant V5.2 software package (GE, Healthcare). Image gel analysis was carried out using the DeCyder 2D Software V6.5 following the manufacturer's instructions (GE Healthcare). The images were exported to the DeCyder Batch Processor module and DIA (Differential in-gel analysis) and BVA (Biological Variation analysis) modules were made automatically. The DIA module was used for spot detection, spot volume quantification, and volume ratio normalization of different samples in the same gel. The BVA module was used to match protein spots among different gels and to identify protein spots that exhibit significant difference. Manual editing was performed in the biological variation analysis module to ensure that spots were correctly matched between different gels and were not contaminated with artifacts, such as streaks or dust. The paired t-test was used for statistical analysis of the data. A false discovery rate (FDR) correction was applied to eliminate false positives. Protein spots that showed a statistically significant change in abundance between ON and OFF samples using a Student's t-test ($p < 0.05$) were considered as being differentially expressed.

2.4.2.4 Protein identification by mass spectrometry (MALDI, MS/MS) analysis

To select spots of interest, gels were first stained with Silver Staining Kit, Protein (GE, Healthcare). Proteins of interest were manually excised from analytical gels and were distained with two 5-min washes with acetonitrile (ACN)/water (1:1, v/v), followed by rehydration with 50 mM ammonium bicarbonate for 5 min and 25 mM ammonium bicarbonate in 50% (v/v) ACN for 15 min. Gel pieces were then digested with sequencing grade trypsin (Promega) as described elsewhere (Shevchenko *et al.,* 1996), and subject to

PMF and/or LC/MS/MS analyses. The digestion mixture was dried in a vacuum centrifuge, resuspended in 7 mL of 0.1% TFA (trifluoroacetic acid, Sigma), and 1 mL was spotted onto the MALDI target plate. After air-drying the droplets at room temperature, 0.5 mL of matrix (5 mg/mL CHCA) (acyano-4-hydroxycinnamic acid, Sigma) in 0.1% TFA-ACN/H2O (1:1, v/v) was added and allowed to air-dry at room temperature. The resulting mixtures were analyzed in a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, USA) in positive reflectron mode (2000 shots each position). Five of the most intense precursors (according to the threshold criteria: minimum signal-to-noise: 10, minimum cluster area: 500, maximum precursor gap: 200 ppm, maximum fraction gap: 4) were selected for every position for the MS/MS analysis. And, MS/MS data were acquired using the default 1 kV MS/MS method. The MS and MS/MS information was sent to MASCOT via the Protein Pilot software (Applied Biosystems). Database searches on NCBI, Swiss-Prot, and HarvESTs: Citrus databases were performed using MASCOT search engine (Matrix-Science). HarvEST: Citrus contains best BLASTX hits from UniProt, the Arabidopsis genome and Phytozome version Poptr1.1 (http://harvest.ucr.edu). Searches were performed with tryptic specificity allowing one missed cleavage and a tolerance on the mass measurement of 100 ppm in MS mode and 0.6 Da for MS/MS ions. Carbamidomethylation of Cys was used as a fixed modification factor, while oxidation of Met and deamidation of Asn and Gln as variable modifications. The samples without a positive identification were analyzed by LC/MS/MS. Peptide separation by LC-MS/MS was performed using an Ultimate nano-LC system (LC Packings) and a QSTAR XL Q-TOF hybrid mass spectrometer (AB Sciex). Samples (5 mL) were delivered to the system using a FAMOS autosampler (LC Packings) at 30 mL/min, and the peptides were trapped onto a PepMap C18 pre-column (5 mm & 300 mm i.d.; LC Packings). Peptides were then eluted onto the PepMap C18 analytical column (15 cm x 75 mm i.d.; LC Packings) at 300 nL/min and separated using a 30 min gradient of 5-45% ACN. The QSTAR XL was operated using an information dependent acquisition mode, in which a 1-s TOF MS scan from 400 to 2000 m/z was performed, followed by 3-s product ion scans from 65 to 2000 m/z on the three most intense doubly or triply charged ions. The MS/MS information was sent to MASCOT via the MASCOT DAEMON software (MATRIX SCIENCE). The search parameters were defined as for MS-MS/MS analysis.

2.4.2.5 Starch analysis

Leaves (2 g) were dried in the oven (60ºC) and then were treated with 80% ethanol. The remaining pellets were gelatinized by autoclaving and then sodium-acetate buffer and amyloglucosidase were added to the gelatinized extracts, according to Schaffer *et al.* (1987). Enzymatic digestions were performed for 2 h at 55ºC. After filtration, released glucose was quantified with a Waters HPLC system equipped with a carbohydrate column (4.6 x 250 mm, 5 mm, Tracer Carbohydrat Tecknokroma, Barcelona, Spain) and a 2410 differential refractometer. A binary isocratic phase consisting in ACN:water 75:25 (v/v) was used and the retention time for glucose was 11.5 min. Quantification was performed by external standard calibration. Starch content was expressed in mg g^{-1} dry weight.

2.4.2.6 Catalase activity

Leaves (2 g) were homogenized in a Polytron 3100 (Kinematica, Lucerne, Switzerland) using 10 mL of 50 mM phosphate buffer, ph 7.0, containing 2mM EDTA and 2% polyvinylpolypyrrolidone (PVPP, Sigma, Barcelona, Spain). The crude extract was centrifuged at 12,000 rpm at 4ºC for 30 min, and the supernatant was filtered (0.45 mm; Nylon) and used for the catalase assay within 1 h. The protein concentration in the supernatant was determined in the TCA precipitate using bovine serum albumin as standard (Lowry *et al.,* 1951). The reaction medium (2 mL) contained 100 mM phosphate buffer pH 7.0, and 100 mL of the supernatant. The reaction was started by adding 100 mL of 10 mM H_2O_2. Catalase activity was spectrophotometrically determined by the decrease in hydrogen peroxide (Tewari *et al.,* 2005). The reaction was monitored at 240 nm in a spectrophotometer UV-1610 (Shimadzu Corp., Kyoto Japan), at room temperature. The molar extinction coefficient used was 43.6 M^{-1} cm^{-1}. Catalase activity was expressed as mmol H_2O_2 consumed g^{-1} protein min^{-1} after 3 min reaction.

2.4.2.7 Gene ontology analysis

Total amounts of isolated protein were analyzed separately in two groups, which were established according to the ratio expression using FatiGO (http://babelomics.bioinfo.cipf.es) (Dimmer *et al.,* 2008). The database at

www.arabidopsis.orgs was used to search for the *Arabidopsis* proteins that were homologous to the proteins identified in this study. Establishing homologies enabled us to understand the main biological processes in which proteins are involved.

2.4.3 Bisulphite treatment

450-750 ng genomic DNA was subjected to one treatment of sodium bisulphite conversion using the EpiTect Bisulphite kit (Qiagen) according to the manufacturer's instructions. The reaction was then purified once more using the PCR purification kit (Qiagen, USA).

2.4.4 Gene expression analysis by qRT-PCR

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 for each experiment. 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	CDS	5′ -Direct primer- 3′ 5′ -Reverse primer- 3′	Predicted product (bp)
ACTIN	Ciclev10025866m	TTAACCCCAAGGCCAACAGA TCCCTCATAGATTGGTACAGTATGAGAC	141
CiFT1	Ciclev10013731m	GGGAGGCAGACTGTTTATGC TCATCGTCTGACAGGCCTTC	150
CiFT2	Ciclev10012905m	TCTAGCAGGGACAGAGATCCT AGAACATCACCAACAACGCG	53
CiFT3	Ciclev10012629m	GGCTGAGGGAGTACTTGCAT TGCCGGAACAACACGAAAAC	139
FLC	Ciclev10033420m	GGCAACTTGAAGGTCCAAAC GCCCAATGAGCATAGGAATG	124
CsLFY	Ciclev10033942m	TCTTGATCCAGGTCCAGAACATC TAGTCACCTTGGTTGGGCATT	63
GA20ox1	Ciclev10005157m	ACCAAGTGGGTGGTCTTCAG TGAAGGTGTCGCCAATGTTA	96
GA3ox1	Ciclev10027153m	CAACGCAAGATGTCAAATGG CAGGCCGGGTAGTAATTCAA	85
SVP	Ciclev10026457m	AGTGGCGGAGGTATCAAATG TGAGGGAGGTGTCTGAGCTT	119
TEM1	Ciclev10031846m	GCAAATGTCTTGTGCTGGAA TGTGCTTCCTCAGCATATCG	104
ELF8	Ciclev10007295m	TCTCGATCTCTGGCTCATCA AGGACTAGACCCTTCCTCCAA	91
SKB1	Ciclev10007631m	GGGTTCGCTGGTTATTTTGA CCGTTGATGGCTCAATACCT	67
12ATX	Ciclev10018602m	TGTGTTGGCTGCCAGTTTAG AATATCCCCAGGCTCGAGTT	52
7ATX	Ciclev10000043m	CGAACACATTTATGCCAACG GCACCCATTAGTGGAGCAAT	70
5ATX	Ciclev10018614m	TAGGGTGAAAGGTTCCATGC CTGCTTCCGCTCCTTCATAG	121

2.4.5 Sequence analysis and phylogenetic trees

CiFT1-3 sequences *(*Nishiwaka *et al.*, 2007; Samach, 2012) and *CsLFY* sequence (Pilliteri *et al.*, 2004) were used for these studies. *FLC* (**Supplementary data S1**), *GA20-oxidase*, *GA3-oxidase*, *SVP*, *TEM*, *ELF8*, *SKB1* and *ATXs* amino acid sequences of *A.thaliana* and other annual plant species and woody evergreen and deciduous tree species were obtained from the NCBI database (www.ncbi.nlm.nih.gov). Sequences were aligned against the *Citrus clementina* genome using the TBLASTN tool of Phytozome v10.3 database (www.phytozome.net). Based on this sequence similarity, some putative homologs of these genes were identified in the *Citrus clementina* genome. The *GA20ox* study was based on the similarity to the characterized amino acid sequences of *CcGA20ox1* and *CcGA20ox2* from the citrus hybrid citrange Carrizo [(*Poncirus trifoliata* Raff. x *Citrus sinensis* (L.) Osb.)]. The *GA3ox*, *FLC*, *SVP*, *TEM*, *ELF8*, *SKB1* and *ATXs* from *A.thaliana*. Phylogenetic trees are given in **Supplementary figures** at the end of

this chapter. Primers used for qRT-PCR analysis (http://frodo.wi.mit.edu/) are listed in **Supplementary Table 1** and primers by PCR analysis (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) are listed in **Supplementary Table 2**.

2.4.6 Generation of constructions in plasmid vectors

2.4.6.1 Polymerase chain reaction (PCR)

The bisulphite treated DNA was amplified using Hot start Platinum® Taq DNA Polymerase (Invitrogen). The thermal cycling program was set at 95°C for 1 min followed by 40 cycles of 95°C for 30 s, annealing 50º for 30 s, and extension at 65-72°C for 30 s, ending with a 3 min extension at 65-72°C.

PCR products were analyzed in agarose gels of 1%.

Forward (F) and reverse (R) primers for bisulphite sequencing PCR were designed using MethPrimer: designing primers for methylation PCRs, given in **Supplementary Table 2**.

Table S2. Primer sequence used in PCR amplification reactions.

Annotation	**CDS**	**5′ -Direct primer- 3′** **5′ -Reverse primer- 3′**	**Predicted product (bp)**
CiFT1	Ciclev10013731m	TTATTTTTTTTATTTATGTTTATTTTTGGTTTTTTTG TTTCTAATTAATCCAAAAAAAAAAAAATAATCACTTAC	112
		TTATATATGTATGTAGGTTAAGAGATTGTG CCCTCTAACAATTAAAATAAACAAC	211
		TTAATTGGTTGTGGTTAGTGATTTTT TAAACAATCTACCTCCCAAATTACC	355
CiFT2	Ciclev10012905m	GTAGGAGGAGGAGGTTTTAGTATTG AAAACAACCTCTCACCTCTAAACAT	278
FLC	Ciclev10033420m	TTGTTAGTATTGTTGTGTTTTATTTTATAT AAAAAACCTAAATAATTATATTCTATTCAT	387
		TTGGGATTATTTTTAAAATTTTGTA AAATTCTCTACTTCTAACAAATCCA	386
		GTTTTGATGAAATATTTTAAAAGTAA ATAAAACTAATCACCTACAATACACC	340
SVP	Ciclev10026457m	TGAGAGGAATAAAGTTTGAAGTAAT AAAAAACACTACAACTCTCCTTAAC	363
TEM1	Ciclev10031846m	TGGGAAAAATTTAAGTTAAAATGTA TTTAATAATTTTATTTTACCACCAC	297

2.4.6.2 Ligation in pGEM-T Easy I vector

PCR products have adenine over-hang that forms by Taq DNA polymerase activity and can bind with thymine sticky ends of the pGEM-T vector. Hence, directly from PCR reaction tubes 2 µl of amplified DNA fragment with 5 µl 2x Rapid ligation buffer for T4 DNA Ligase, 1 µl of linear pGEM-T vector and 1 µl T4 DNA ligase (3U/µl) (Promega) were mixed and then incubated at 37ºC for 1 hour (alternatively at 4ºC for 24 hours).

2.4.6.3 Blue-white screening

E.coli cells, which are transformed with pGEM-T vectors, have an ampicillin resistance gene and a LacZ operon which encodes a β-galactosidase. LacZ expression is induced by IPTG, a lactose analogue that cannot be metabolized by *E.coli*. LacZ releases indole from the substrate X-gal (a glycoside composed of galactoside and indole). Indole molecules form blue dimers. When pGEM-T vectors contain an insert in the cloning site, the *LacZ* gene is interrupted and no color will be produced. So blue colonies indicate re-closure of plasmid DNA resulting in *LacZ* gene function, whereas white colonies indicate that the DNA of interest was inserted into the plasmid and caused interruption of the *LacZ* gene. For blue-white screening 1 ml of grown cells were poured on LB agar plates. Plates were surface dried in the laminar air flow and incubated at 37ºC over night. Then, the white colonies were collected and grown in 5 ml LB + Ampicillin over night at 37ºC.

2.4.6.4 Digestions

DNA of interest with the plasmid was digested with EcoRI enzyme for 2-3 hours at 37ºC. Then 6-12 individual clones were sequenced.

2.4.6.5 Cloning *FLC* into a plant cloning vector

FLC codification region was amplified by PCR. The cloned was obtained from IVIA1 library, IC0AAA56AF11. Subsequently, it was cloned in pCR8 and after that, all of this was introduced in pEarlygate 201 (kanamycin and glufosinate resistant). Next, this construction was introduced in *Agrobacterium* (gentamicine and rifampicin resistant).

2.5 Hormone isolation, purification and quantification

Material frozen in liquid nitrogen was ground into a fine powder. Aliquots (about 50 mg fresh and 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, IP, Tz and JA 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 RP-MS 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.

2.6 Evaluation of leaf number

To evaluate the effect of citrus *FLC*. Three lines of *Arabidopsis thaliana* cv. Columbia were transformed. The first was control (col) and the second line was introduced the *FLC* of citrus (col-CcFLC). When all of these plants flowered the number of rosette and caulinar leaves was evaluated.

2.7 Statistical analysis

Parameters were statistically tested by analyses of variance (ANOVA), using the least significant differences (LSD) test for means separation. The experimental data were analyzed with Statgraphics Plus 5.1 software (Statistical Graphics, Englewood Cliffs, NJ).

2.8 Supplementary figures. Phylogenetic trees

Figure S1. Phylogenetic analysis of GA20 oxidases in *Citrus clementina*. Only the CDS relationship with GA20 oxidase 1, Ciclev10005157m, was selected according to its similarity with citrange Carrizo.

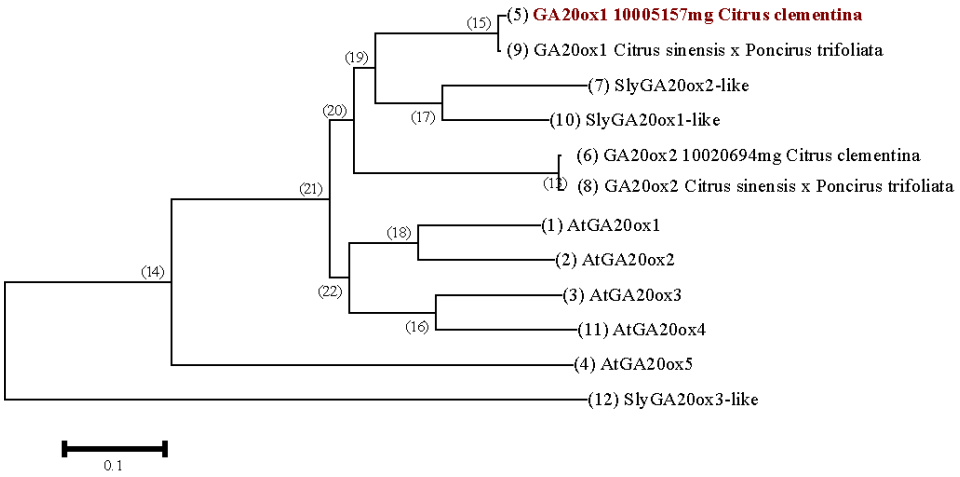


Figure S2. Phylogenetic analysis of GA3 oxidases in *Citrus clementina*. Only the CDS directly related with GA3 oxidase of *Solanum lycopersicum* and with the GA3 oxidase 1 of *Arabidopsis thaliana*, Ciclev10027153m was selected.

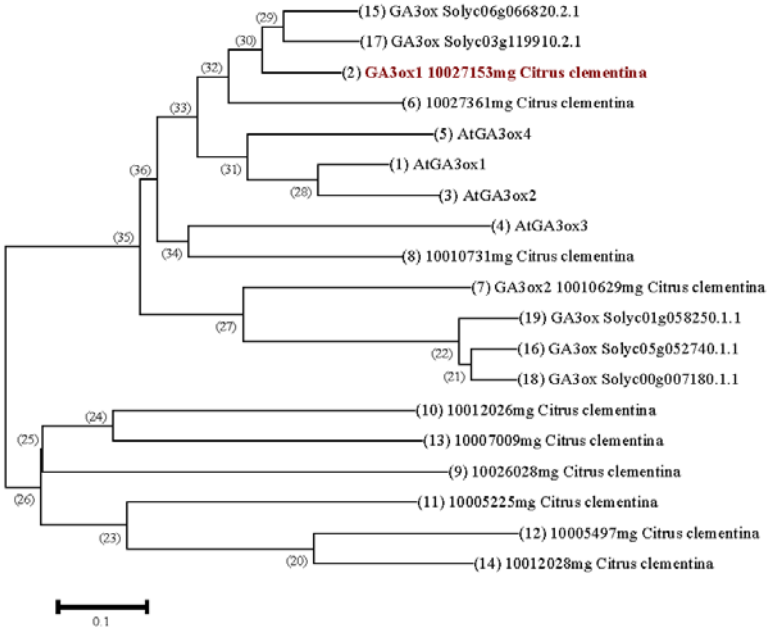


Figure S3. Phylogenetic analysis of FLC in *Citrus clementina*. Only the CDS inside the FLC/MAF branch was selected, it was directly related with FLC of *Poncirus trifoliata* and *Vitis vinifera*, Ciclev10033420m.

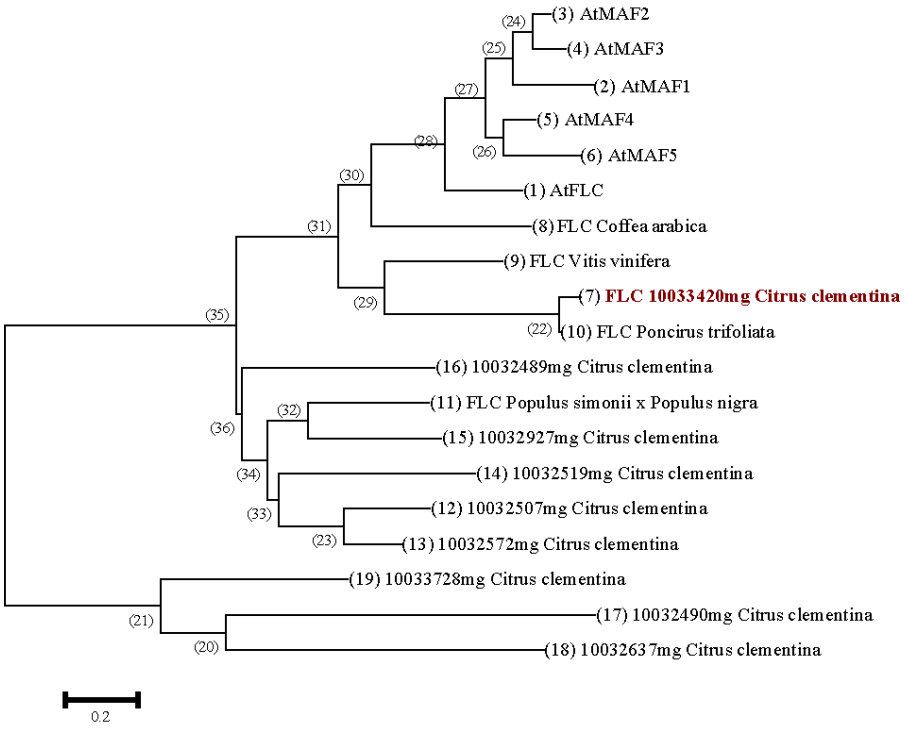


Figure S4. Phylogenetic analysis of SVP and TEM in *Citrus clementina*. Only the CDSs in the middle of the SVPs and TEMs, respectively, of each branch, Ciclev10026457m (SVP) and Ciclev10031846m (TEM) were selected.

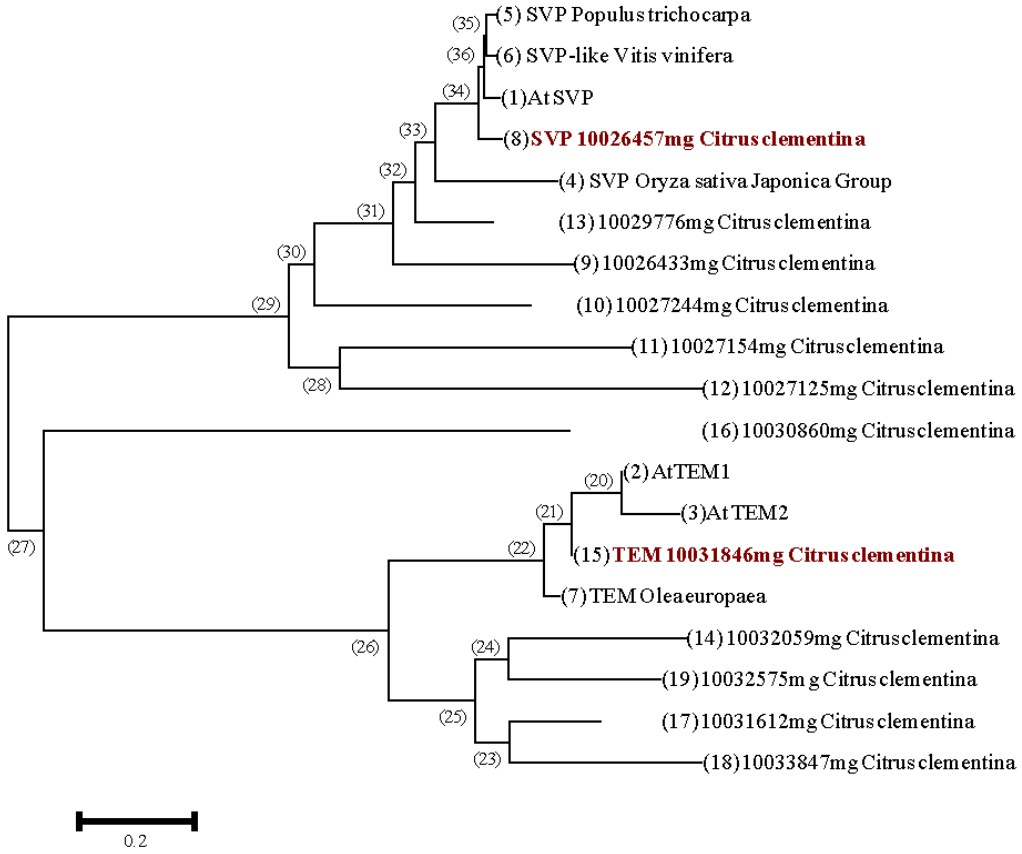


Figure S5. Phylogenetic analysis of ELF8 and SKB1 in *Citrus clementina*. Only the CDSs inside the branch of the ELF8 and SKB1, respectively, Ciclev10007295m (ELF8) and Ciclev10007631m (SKB1) were selected.

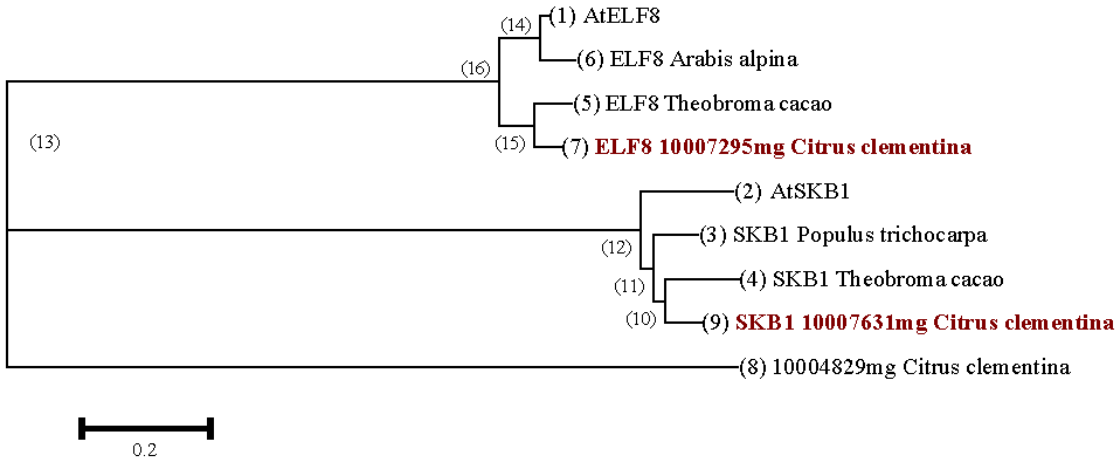
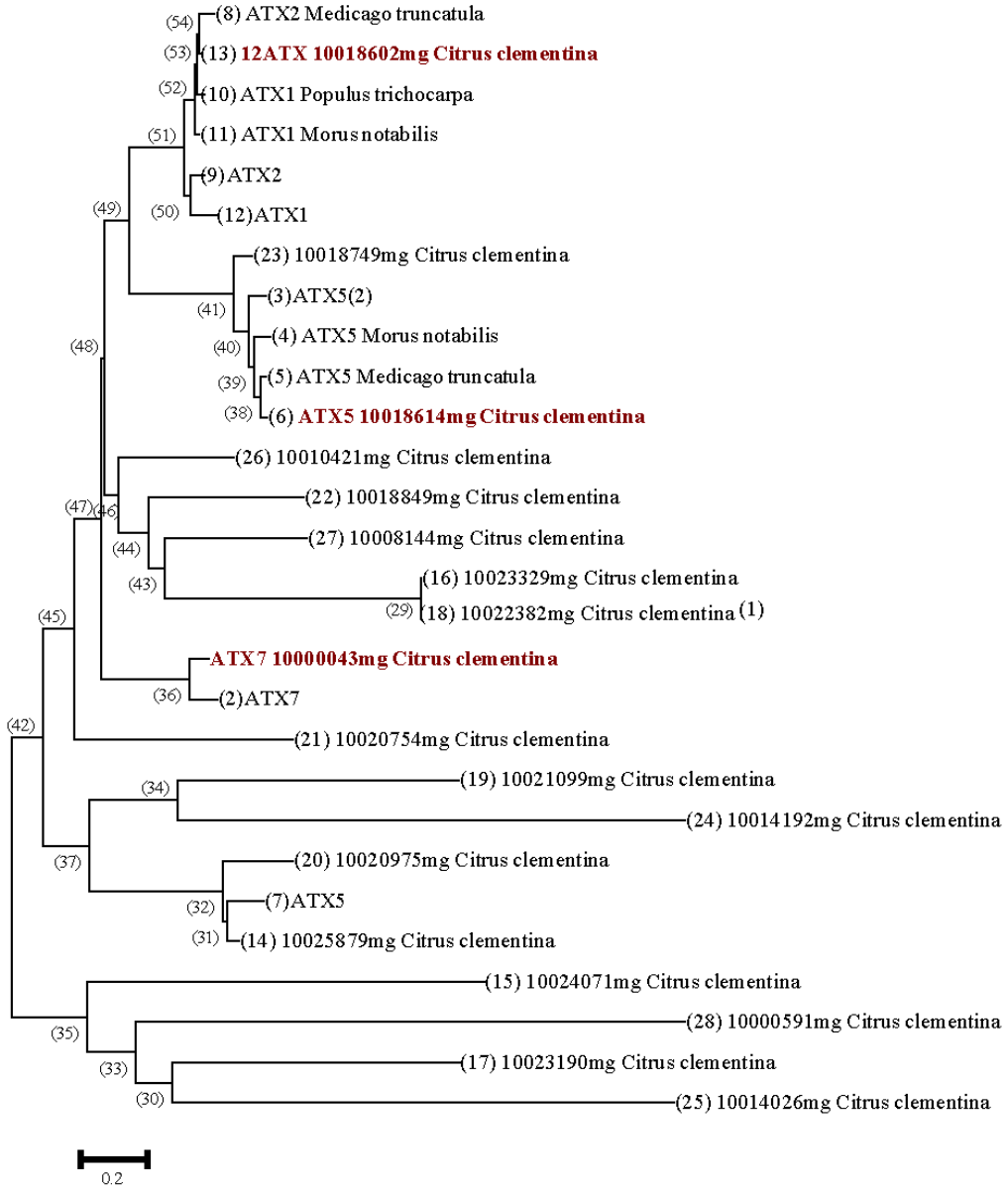


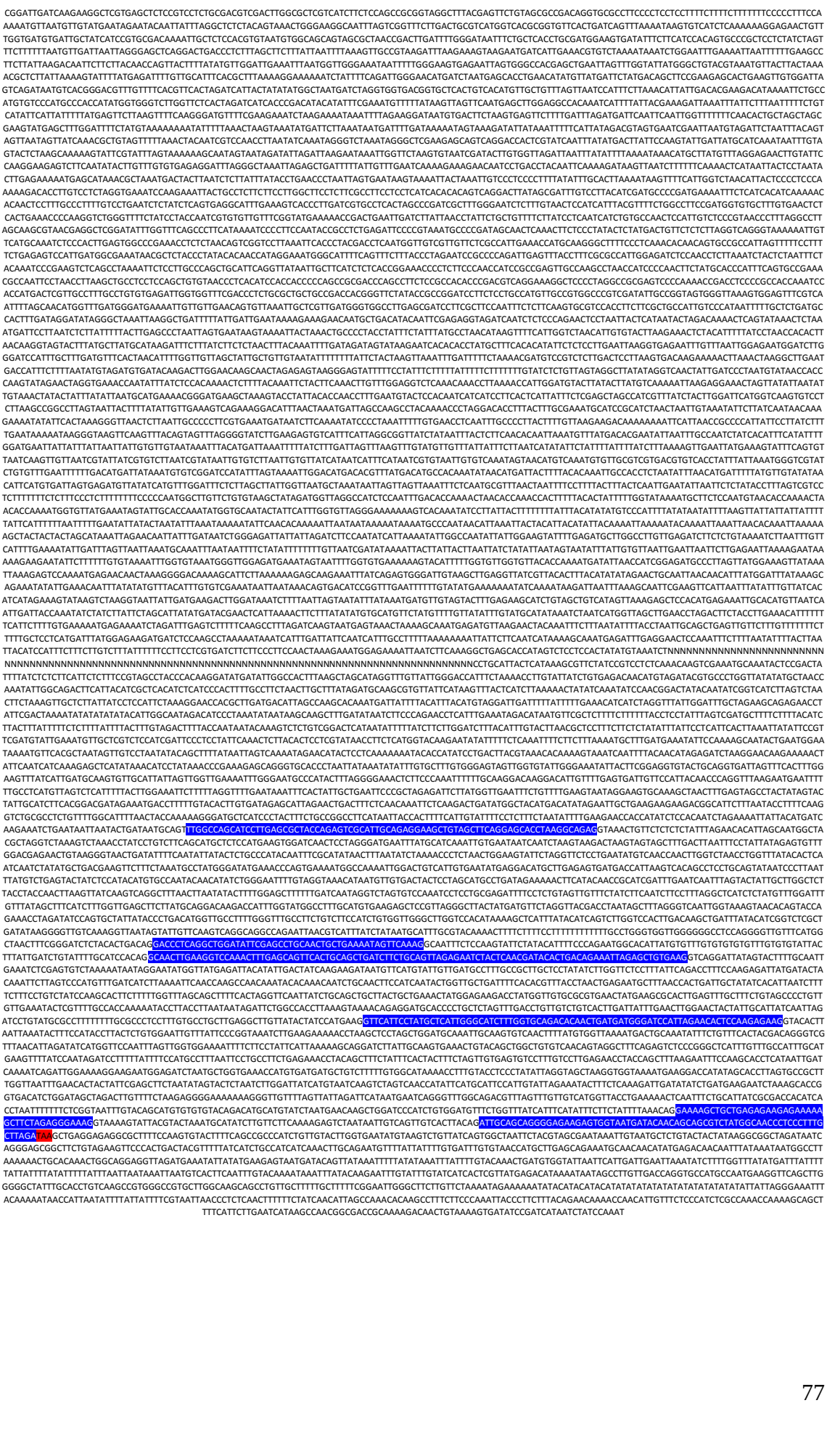
Figure S6. Phylogenetic analysis of 1, 2, 5 and 7ATX in *Citrus clementina*. Only the CDSs inside each branch of the 1, 2, 5 and 7ATX, respectively, Ciclev10018602m (1,2ATX), Ciclev10018614m (5ATX) and Ciclev10000043m (7ATX) were selected.



2.9 Supplementary data. *FLC* sequence

Data S1. Genomic sequence of *FLC*, obtained of IVIA1 library, IC0AAA56AF11 clone. In Phytozome v10.3, it corresponds with Ciclev10033420m.g.

TTTATAATTTTATAATATATATGTAGATATATGAAATTTTATTAAATATAAAAATAATTATCACTATAAGATGTGATAATTTTATAAAAAGTAATTTATAAAAAATTTATCAAATATCAAAATTAACTTTTAACTTTTAAACATAATAATTGAGCTT
CTAAAAAATTAATTCCAAACGGCCTTAATGTATGAATAATTTTTTTTTTAATTACAAAATATATATTTTTATTAAATAATTCAATCATCAAAAAAGTTTTGCTGTCTTAGGAGAATTAATTTGTAGGACCCACAGGCGCGTACACGAAAAAAATG
ATAAAATGAGACTCAGGTCTATCTGCAGTAATAAACTAAGCTAACATGTTGACTTGCAAAAAGTTAATTTATTTGGTGTCGGCACTTAAGTGATTCCAGTAGAATAATTATACATCAAATTTGGAGGTTTGACGTGAGAAGACATTGGCTGGC
CTTGGCTGGTTAATCTGGTAACTTACTTTAATCATACAGTTTATCCACTAGCCACTGGGTCCTATCGCAACAAAATGTTGTTTTTATTTTTAAAAAAATAAACAAAAGGGTGTAAACAAGTTCACTCTTTCTAATTAGAGTAATTTATTTGTTTCT
AAAAAGAGTAATCTAATCTTTCTTCGAAATAAAATAATTAATAAATAAATAAATAAATAAATAAACTAGGCGTGCCGAAAGTCCCCCAAAAAAAAAAAAACCTTAAAAATACGACAACAAATAACACGCACGGCACACGTAGCATTAAAAA
ACAAATAAAAAAGCAGCCGCAAGCCCGCAACAAAAGCACGAATGATGCCCGCGGAGACACGTGTCGAATTGTCAGGCGTCCTCGCCTCCGACTTTCGATTGATCGATTTATTCACACACTGTCAGCACTGCTGTGCTTTACTCTACATAAGT
ACCACCAAGCGACCCTAATTTCCGCCTTATCTGCTGTGCTCCTTTTCTCAATCTAGGGCGGCATTGGGTCAGAAATGGCCGGAAAAAGCTTCAACTGCAGCGAATCGAAAACAAAAGCCGATGTCAAGTAACGTTCTCAAAGCGGCGTAG



Results

Section 1. Hormonal inhibition of flower induction: Gibberellins

In citrus, when it reaches its final size fruit inhibits flower induction by repressing the expression of flowering genes. But it is not known which long (*or short*) distance signal fruit sends produces to hamper the process. GAs have been thought to be this signal. But a "directed" transport of GAs from the fruit to the bud or the leaf has not yet been clearly demonstrated. Besides, the relationship between endogenous GA and flower induction it is not clearly known. The objective of this section is to discuss the effect of fruit in endogenous GA synthesis and transport and flowering.

The 'Moncada' mandarin tree presents an absolutely natural 100% alternation between flowering and fruiting. Thus, it was selected as a model system to study the process. The tree produces up to 80-90 kg year^{-1} (ON-tree). The fruit ripens in January and harvest-time lasts until February. Thereafter, in spring, the tree is not able to flower and only develops vegetative shoots (**Fig. 1.1**). Therefore, the tree does not produce a fruit during a complete year (OFF-tree). Flowering is induced in autumn-winter, and the following spring the tree sprouts and flowers profusely, developing mostly generative shoots (inflorescences), but also vegetative shoots and mixed shoots with flowers and leaves (**Fig. 1.1**). A large number of these flowers set a fruit, the tree becoming ON again.

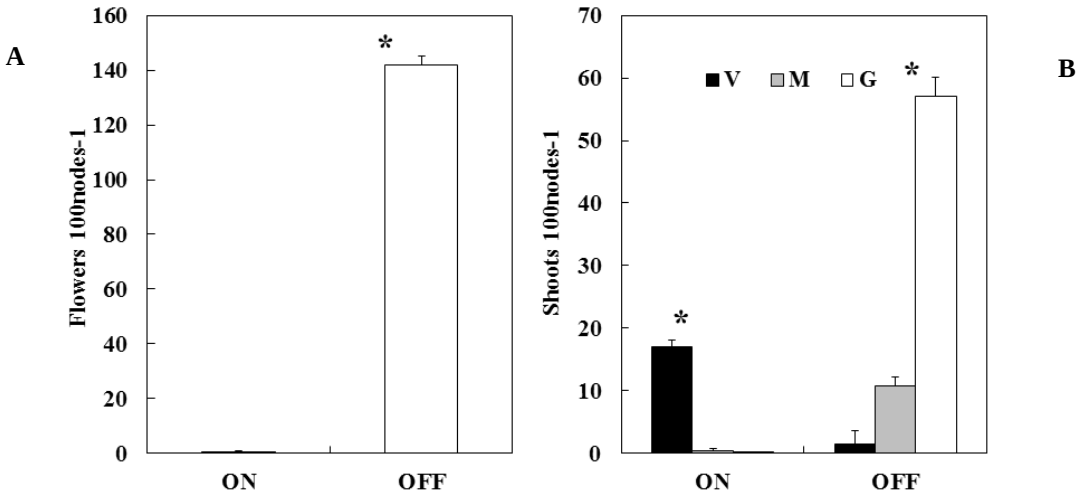


Figure 1.1 Flowering of 'Moncada' mandarin ON (with yield) and OFF (without yield) trees. **A:** flowering intensity. **B**: type of sprouted shoots. V: vegetative shoot; M: mixed-type shoot (flowers and leaves); G: generative shoots (inflorescences or single flowers). Data are means ± ES of 6 trees. *: indicate significant differences ($p \leq 0.05$).

1.1 Fruit development and flower induction gene expression

To study flower induction, *FT* gene expression (main promoter) and *FLC* gene expression (repressor) were determined. *FT* gene has been described as the "florigen" in several species, *Citrus* included (Nishikawa *et al*., 2007). But it has also been reported to regulate both vegetative and reproductive development in trees due to the presence of at least 2 paralogs (*FT1* and *FT2*) (Hsu *et al.*, 2011). In *Citrus*, there are 3 paralogs encoding this type of protein, *CiFT1, CiFT2, CiFT3*. Their role regulating vegetative or reproductive development has not yet been demonstrated. To determine which of these paralogs are related to flower induction, a preliminary experiment was performed comparing *CiFT1, CiFT2* and *CiFT3* expression in juvenile trees (without the ability to flower) and adult OFF-trees. The relative expression was studied in September, November and February, coinciding with the fall sprouting, the floral induction stage and spring sprouting, respectively (**Fig. 1.2**). *CiFT1* did not differs significantly in the juvenile and adult trees, showing increased expression in fall and spring sprouting stages, and reduced expression in the floral induction stage. On the other hand, *CiFT2* was not expressed in juvenile plants in the period studied, while in adult trees the relative expression of *CiFT2* was increased 12-fold in the floral induction stage and 35-fold during flowering. The relative expression of the *CiFT3* gene showed no variation in adult trees and a significant decrease over time in juvenile trees. In summary, only *CiFT2* showed significant differences in the floral induction period between adult and juvenile plants, and, accordingly to this, it may regulate flower induction in *Citrus*. Additionally, *CiFT1* correlated with sprouting in juvenile and adult trees, suggesting a role in the control of vegetative growth, as occurs in poplar (Hsu *et al.*, 2011).

The citrus *FLC* gene has been sequenced (**Supplementary data S1**) and characterized. Thus, the *FLC* gene was introduced in *Arabidopsis thaliana* to study the delaying effect on flowering time. This was evaluated by counting the leaves at the moment of flowering (**Fig. 1.3**). *Arabidopsis* plants transformed with Cc*FLC* had more leaves than the *Arabidopsis* control plant, which indicates that *CcFLC-Arabidopsis* plants presented delayed flowering.

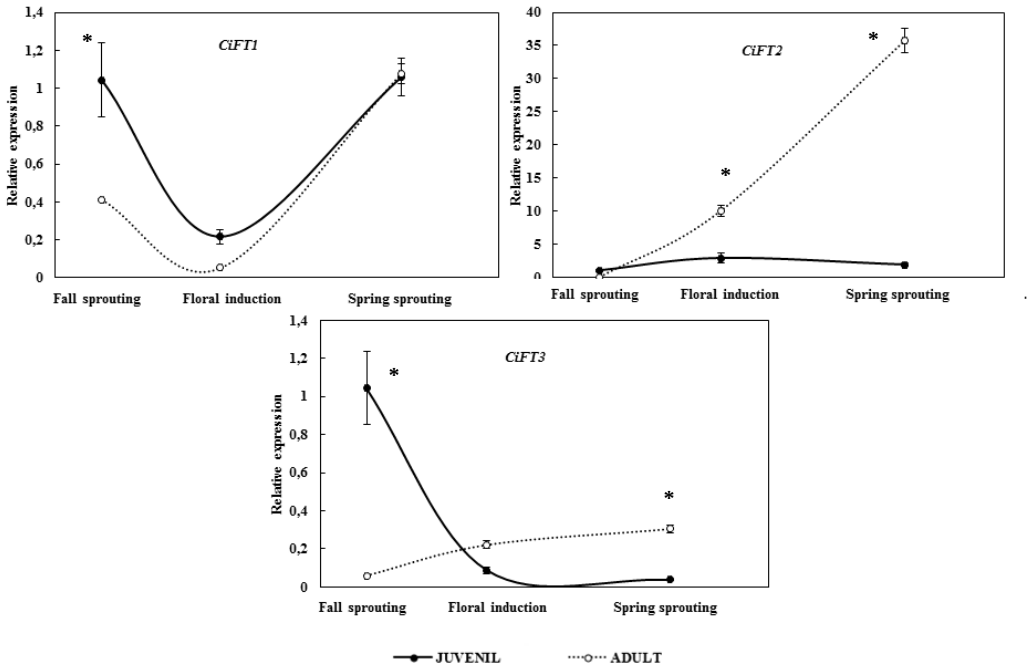


Figure 1.2 *CiFT*, *CiFT2* and *CiFT3* expression in leaves of 'Moncada' mandarin adult trees and leaves of a juvenile plant of 'Cleopatra' mandarin. Data are means ± ES of 3 qRT-PCR replicates. *: indicate significant differences ($p \leq 0.05$).

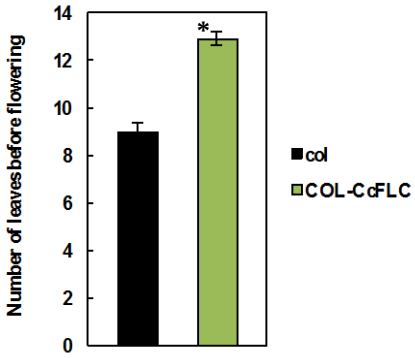


Figure 1.3 Number of leaves before flowering of *Arabidopsis thaliana* cv. Columbia. Influence of the *FLC* gene expression. Data are means ± ES of 40 plants by column. *: indicate significant differences ($p \leq 0.05$).

From the beginning of July, 'Moncada' mandarin fruit underwent a phase of rapid development to reach its final size around mid-November. From the end of September until mid-November, the fruit grew to about 20% of its final size (**Fig. 1.4A**). This period still coincided with high temperatures (**Fig. 1.4B**) and in September and October the color of the peel remained dark green (**Fig. 1.4A**). Thereafter, the end of fruit growth coincided with the beginning of fruit degreening, and chlorophyll content significantly declined (from 350 mg/g PF in October to 110 mg/g PF in January) (**Fig. 1.4C**). The color of the peel changed progressively from pale green to orange a/b (Hunter) around the end of November (**Fig. 1.4A**) coinciding with a significant reduction in temperatures (**Fig. 1.4B**). The distinctive orange color of the fruit was gradually developed until late January (**Fig. 1.4A**). This effect was produced by the increase in carotenoid contents (from 205 1000 OD 440 at the end of November to 260 1000 OD 440 at the end of January) (**Fig. 1.4D**).

The end of fruit growth and the beginning of fruit ripening, due to the decrease in temperature, coincided with the upregulation of *FLC* and the inhibition of *CiFT2* . On the other hand, *CiFT2* was significantly upregulated and *FLC* downregulated in the leaves of the OFF trees (**Fig. 1.4E and F**).

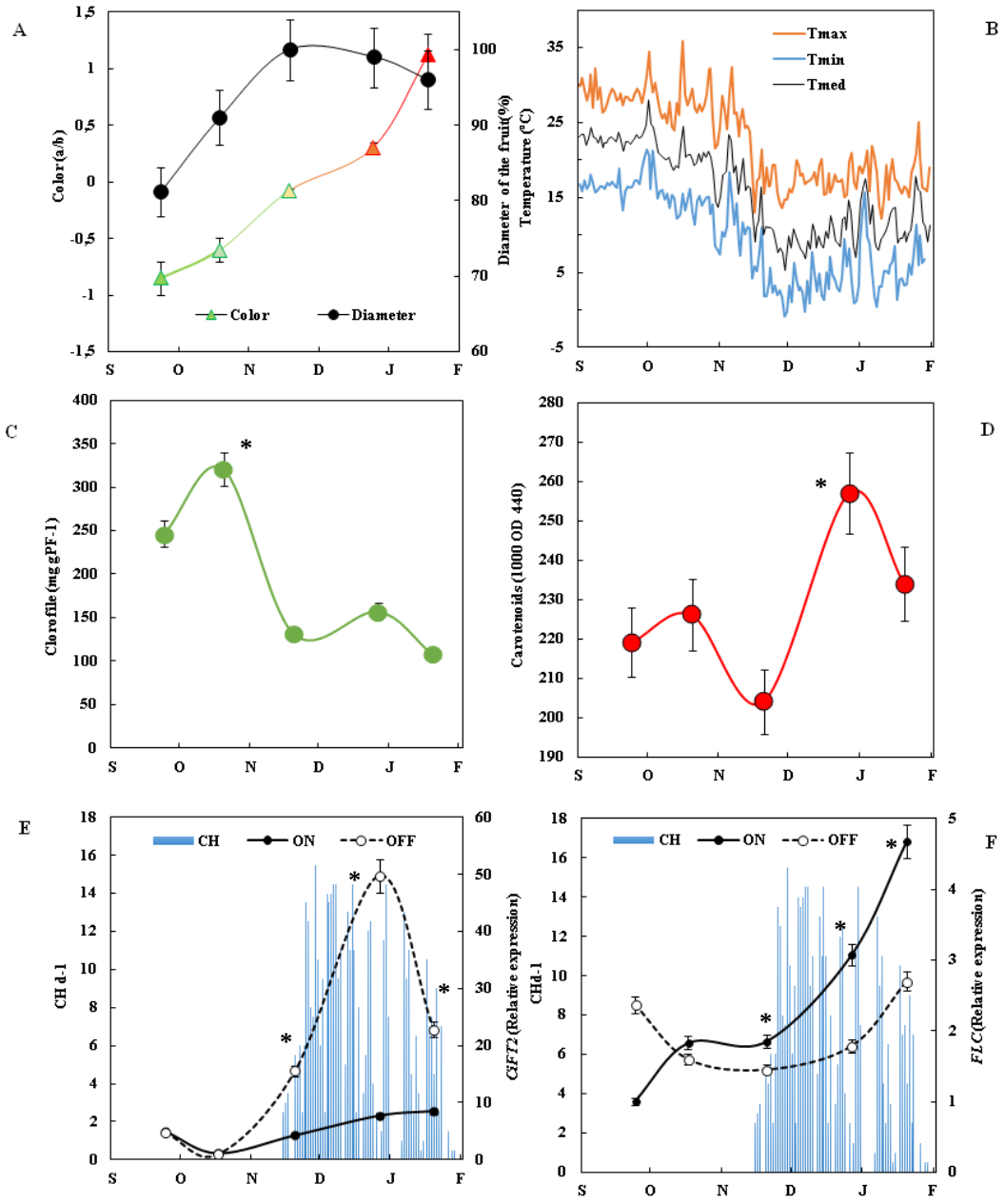


Figure 1.4 Evolution of fruit diameter and the color of the peel (a/b Hunter) (**A**), air temperature (**B**), chlorophyll content in the peel (**C**), carotenoid content (**D**) and *CiFT2* and *FLC* expression related to chilling hours (**E-F**) in ON and OFF trees of 'Moncada' mandarin. The data of fruit diameter and color are the means ± SD of 10 replicate samples. The data for chlorophyll contents are the means ± SD of two measurements. The data of gene expression are means ± ES of 3 qRT-PCR replicates. *: indicate significant differences ($p \leq 0.05$).

1.2 Time-course of gibberellin metabolism in the exocarp, nodes and leaves from ON and OFF trees

Higher concentrations were formed for gibberellins from the 13-hydroxilation pathway than gibberellins from the non-hydroxilation pathway in both ON and OFF trees (**Figs. 1.5 and 1.6**). In ON trees, the beginning of chlorophyll breakdown in the flavedo coincided with a significant reduction in GA_{12} GA_{19}, GA_{20} and GA_{29}. Concentrations of GA_{12}, GA_{20} and GA_{29} rapidly reduced from 0.82 ng g^{-1}, 0.88 ng g^{-1} and 0.81 ng g^{-1} in September to 0 in October. GA_{19} concentration dropped progressively from 2.47 ng g^{-1} in September to 0 in January (**Fig. 1.5**). Neither GA_1 or GA_8 was found in the flavedo. Gambetta *et al.* (2012) suggested that citrus fruit might export GAs to change color, which is in agreement with these results. Concomitantly, GA_{20} and GA_1 concentrations significantly increased (4-fold) in the leaves during November, as well as the GA_{20} catabolite, GA_{29} . The GA_{20} precursor, GA_{44}, also followed this trend although reaching a significantly lower concentration (**Fig. 1.5**). This increase in GAs concentration was not supported by a significant increase in GA synthesis, as deduced from the analysis of *GA20ox1* (which catalyzes the GA_{52}-GA_{44}-GA_{19}-GA_{20}) or *GA3ox1* gene expression in the ON-tree leaves during October and November (**Fig. 1.7**). Although these experiments do not demonstrate the transport of GA to the leaves, it might be suggested, according to the results. On the other hand, GA concentration in the leaves from the OFF trees was high in September and low in November. Specifically, in the youngest leaves (coming from the fall flush), GA_{12}, GA_{19} and GA_1 concentrations fell from 19 ng g^{-1}, 2.1 ng g^{-1} and 2.9 ng g^{-1} in September to 0 in October, being almost nil until the end of the study (**Fig. 1.6**). Accordingly, *GA20ox1* and *GA3ox1* gene expression also peaked in September and progressively diminished until the end of the study (**Fig. 1.7**). Leaves from the spring flush showed a similar trend for these GAs but at lower concentrations. The highest GA synthesis shown in September may be due to the development of the leaves of the fall flush. A detailed representation is shown in **Figure 1.8**. The growing leaves from the fall flush had higher GA_1 and IAA concentrations than the adult leaves from the spring flush. Moreover, these adult leaves supported the growth of the young leaves, i.e the adult leaves of a node with a growing shoot had higher GA_1 and IAA concentrations than the adult leaves of a node without a growing shoot (**Fig. 1.8**).

In the nodes, GA_1 concentration followed the same trend in ON and OFF trees being slightly higher in ON trees (1.71 ng g^{-1}) compared to OFF trees (1.10 ng g^{-1}) (**Figs**

1.5 and 1.6). No GA synthesis was found during the dormant period of the buds, which suggests that GA_1 found in the node is the result of GA-synthesis and transport from other organs (i.e. leaf or fruit). On the other hand, *GA20ox1* and *GA3ox1* gene expression was triggered at the bud sprouting stage (**Fig. 1.7**)

Although the non-hydroxilation pathway is quantitatively less important in *Citrus*, it is interesting to note the time-course of GA_4 concentration in the fruits from ON trees. GA_4 increased from September to October and remained constant at a concentration of 2.3 ng g^{-1} until the fruit color was complete. When carotenoid synthesis was triggered, GA_4 was depleted (**Fig.1.5**).

In brief, in this experiment in fruiting shoots from ON trees, a peak in GAs was found in the leaves when *FT* gene expression was hampered. This also coincided with the moment the fruit reached its maximum size and began the ripening stage. GAs in the fruit were also depleted; GA_1 was continuously found in the node and GA synthesis in the leaf did not increase. Therefore, results might suggest a *short-distance transport* from the fruit to the proximal nodes and leaves. Three major questions arise: 1) Is this GA increase responsible for *FT* inhibition in ON-trees? 2) If GA synthesis is inhibited, can the buds from ON-trees flower? 3) If transport between the fruit and buds is interrupted, can buds flower?

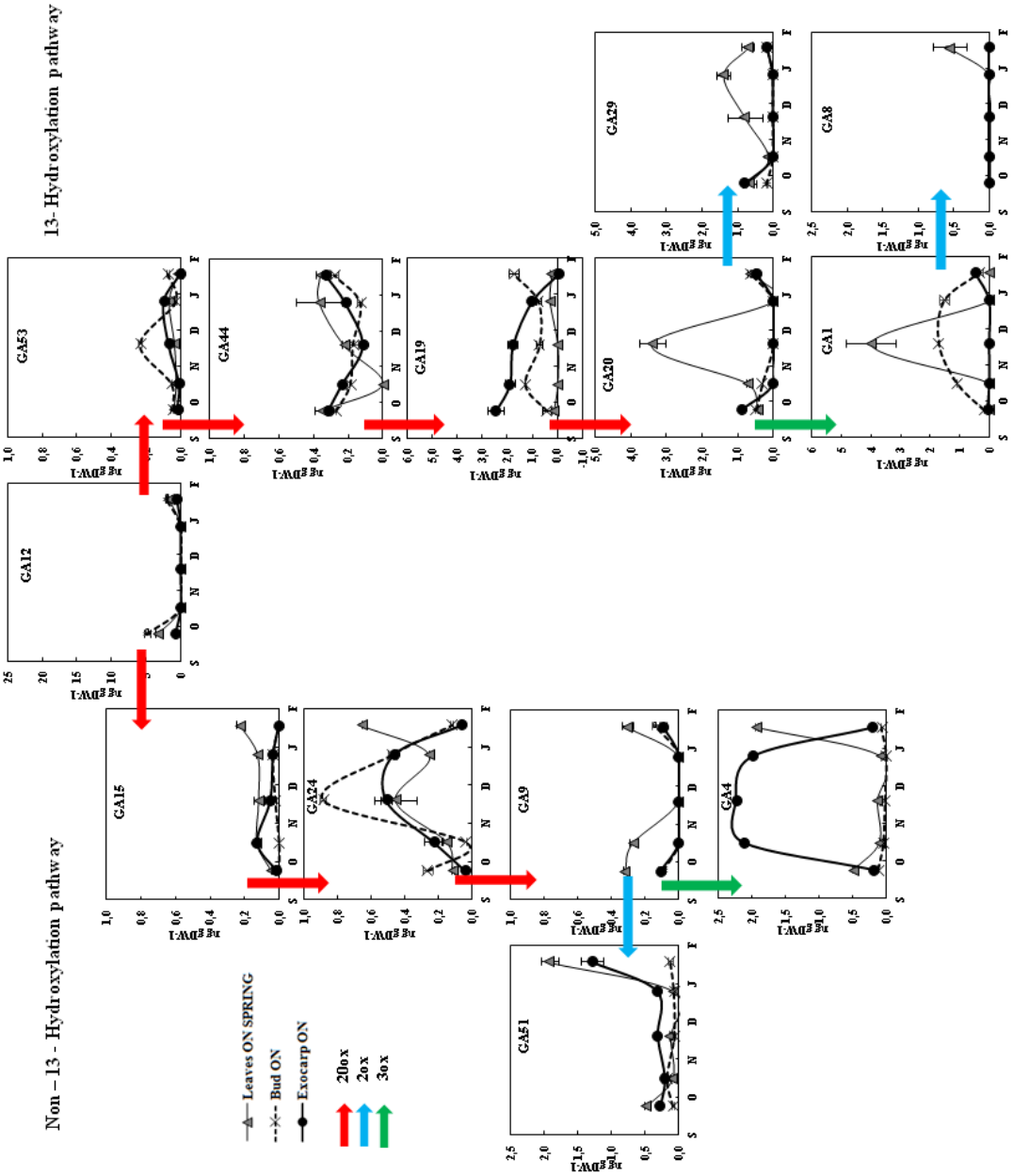


Figure 1.5 Endogenous gibberellin content of the non-13-hydroxylation pathway, (GA_{12}, GA_{15}, GA_{24}, GA_9, GA_4, and catabolite GA_{51}), and 13-hydroxylation pathway, (GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1, and catabolites GA_{29}, GA_8), in ON trees of 'Moncada' mandarin. GAs were measured in the spring leaves, buds and exocarp. Date are means ± ES of two sets of 10 single flowered leafy shoots.

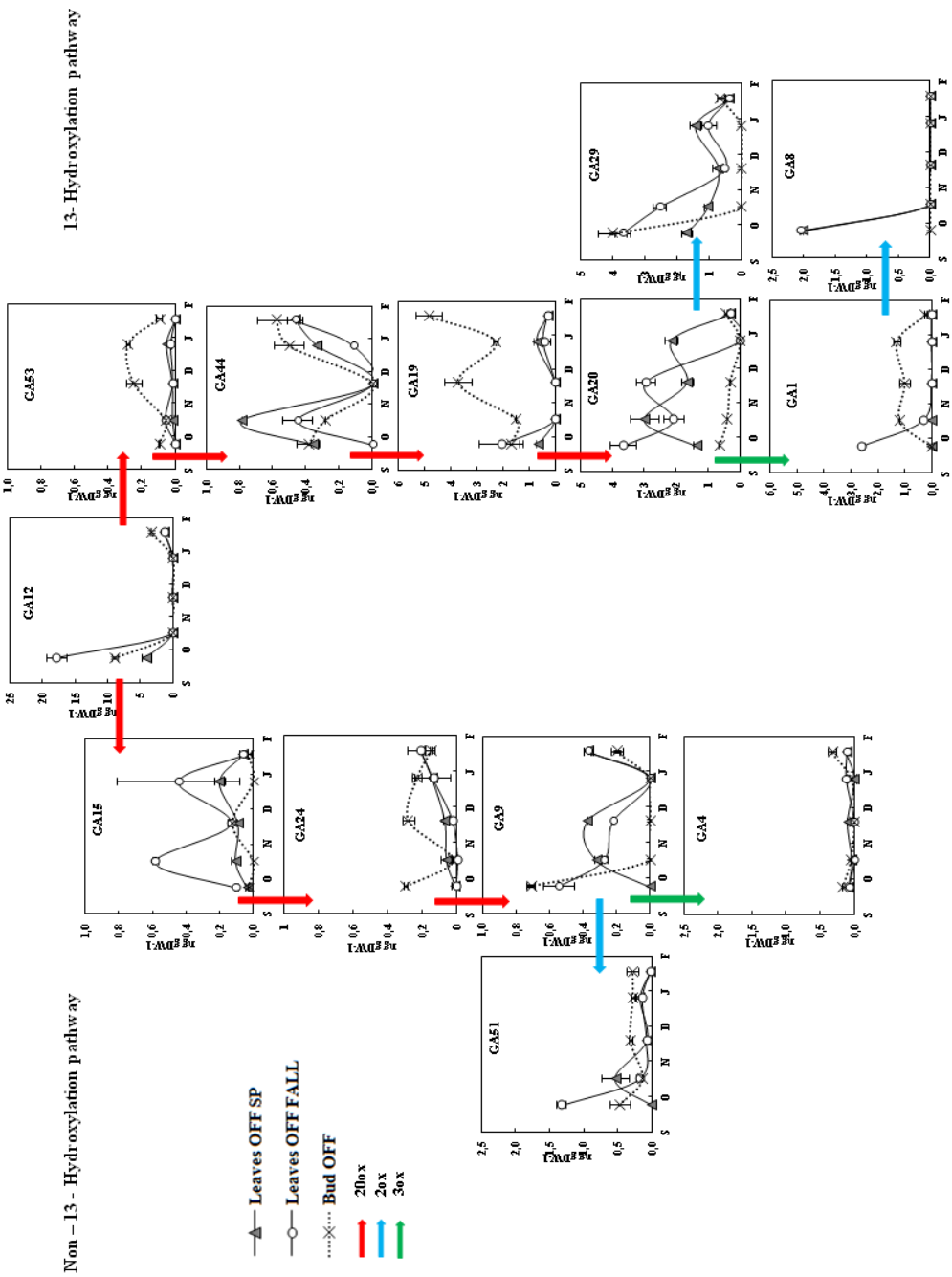


Figure 1.6 Endogenous gibberellin content of the non-13-hydroxylation pathway, (GA_{12}, GA_{15}, GA_{24}, GA_9, GA_4, and catabolite GA_{51}), and 13-hydroxylation pathway, (GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1, and catabolites GA_{29}, GA_8), in OFF trees of 'Moncada' mandarin. GAs were measured in the spring leaves, buds and exocarp. Date are means ± ES of two sets of 10 single flowered leafy shoots.

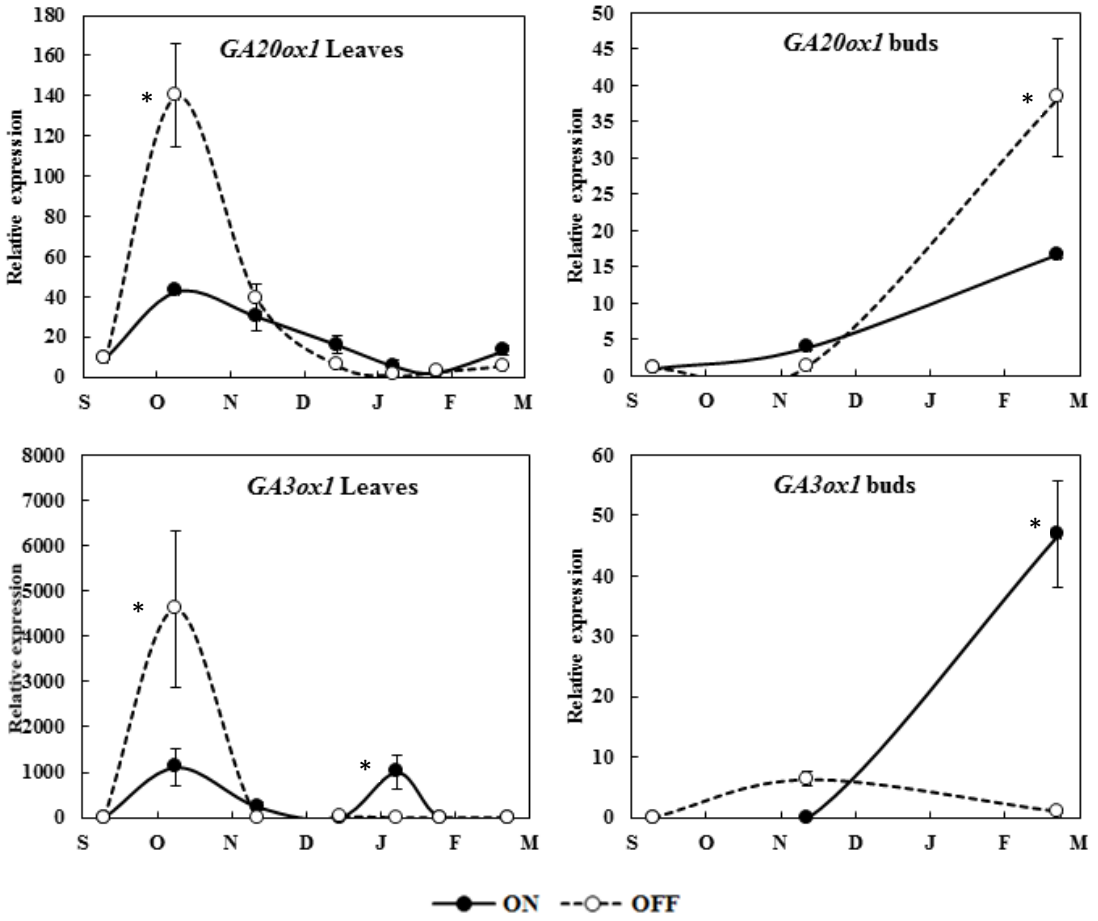


Figure 1.7 *GA20ox1* and *GA3ox1* expression in 'Moncada' mandarin leaves and buds from September to February. Data are means ± ES of 3 qRT-PCR replicates. *: indicate significant differences ($p \leq 0.05$).

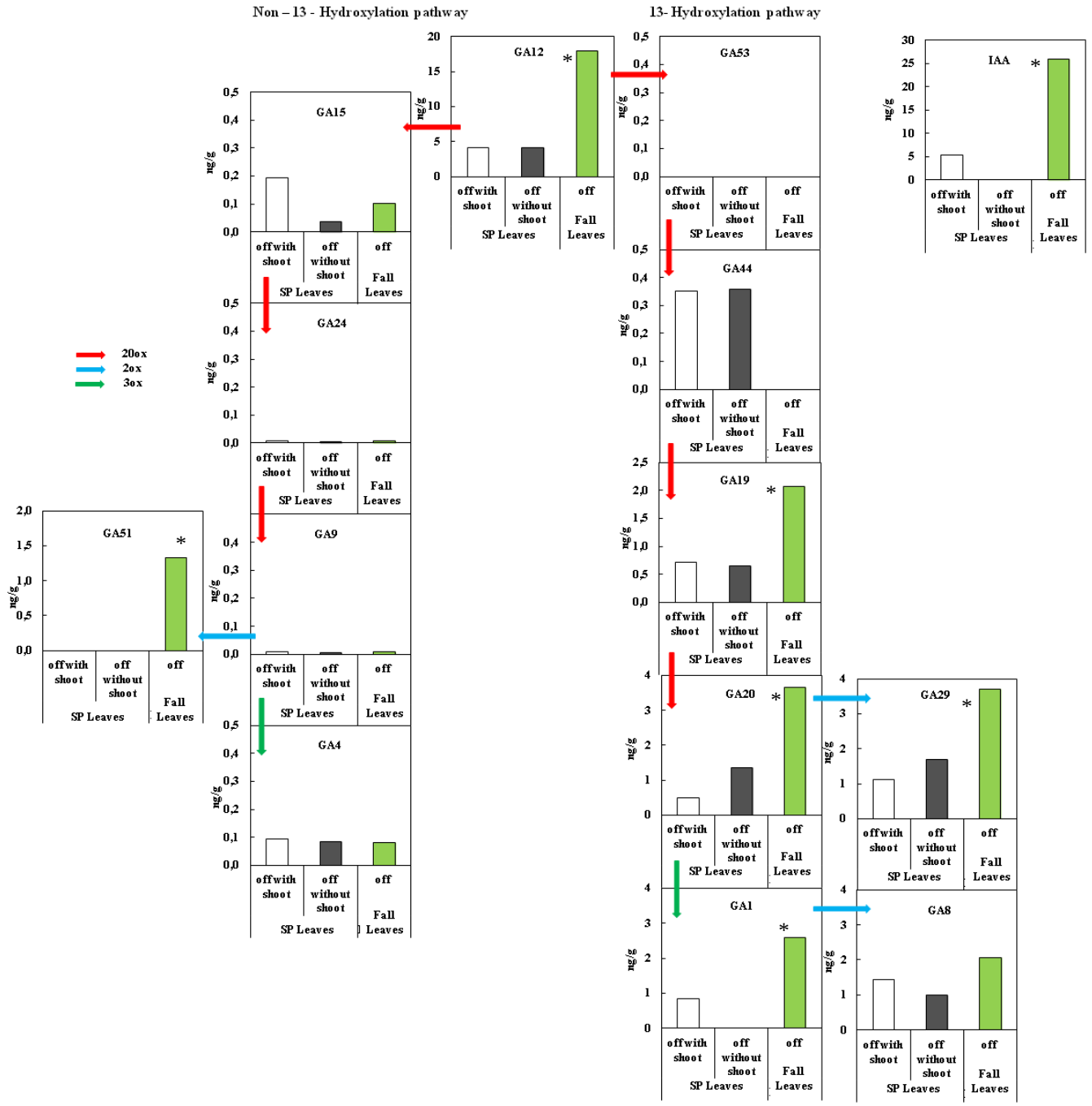


Figure 1.8 Endogenous gibberellin content of non-hydroxylation pathway, GA_{12}, GA_{15}, GA_{24}, GA_9, GA_4, and catabolite GA_{51}, and 13-hydroxylation pathway, GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1, and catabolites GA_{29}, GA_8, in OFF trees of 'Moncada' mandarin. GAs were measured in the spring (SP) adult leaves from nodes with and without new fall shoots, and in fall leaves. Dates are means ± ES of two sets of 10 vegetative shoots. *: indicate significant differences ($p \leq 0.05$).

1.3 Exogenous control of flowering: Treatments with Paclobutrazol (PBZ) and GA_3

Medium-yield trees

PBZ (1 g tree^{-1}) applied at the flower induction period significantly increased flowering intensity (70%) in 'Hernandina' Clementine mandarin (70 flowers per 100 nodes) as compared to untreated trees (40 flowers per 100 nodes) (**Fig. 1.9**). Doubling the amount of PBZ applied (2 g tree^{-1}) did not improve the response. Gibberellic acid had the opposite effect as the number of flowers per 100 nodes dropped by 37%, from 40 (untreated trees) to 25 flowers per 100 nodes (50 mg l^{-1} GA_3 treated-trees) (**Fig. 1.9**); the effect of GA_3 prevailed over that of PBZ when the two regulators were applied together (**Fig. 1.9**).

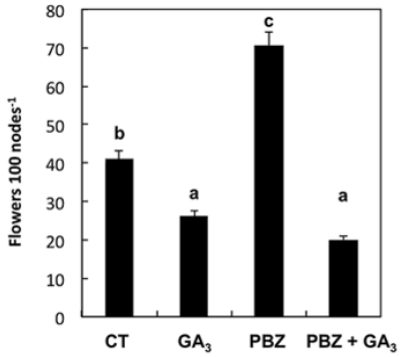


Figure 1.9 Effect of paclobutrazol and gibberellic acid applied to 'Hernandina' Clementine mandarin. Values are the means of 10 trees per treatment. Standard errors are given as vertical bars.

This effect does not depend on the species. Thus, the application of 40 mg L^{-1} of GA_3 at the flower bud inductive period also reduced the number of flowers per 100 nodes of 'Salustiana' sweet orange by 72% ($p \leq 0.01$) in comparison to control trees (**Table 1.1**). This treatment also reduced bud sprouting by 40% ($p \leq 0.05$) compared to the control. Leafless single-flowered shoots and leafless inflorescences were reduced on average from 3.6 to 1.3 and from 5.1 to 1.9 per 100 nodes, respectively, due to treatment, with differences being statistically significant ($p \leq 0.05$). Among flowered leafy shoots, only inflorescences were significantly reduced by application GA_3 from 5.6 to 1.2 per 100 nodes ($p \leq 0.05$). Conversely, GA_3 significantly increased vegetative shoots from 3.8 to 9.0 ($p \leq 0.05$). PBZ applied at a concentration of 2,000 mg L^{-1} produced an opposite

trend. The number of flowers per 100 nodes and percentage of sprouted buds were increased by 123% and 74%, respectively, compared to the control ($p \leq 0.05$). For leafy shoots, neither single-flowered nor inflorescences were significantly altered by this treatment. However, for the leafless shoots, both single-flowered and inflorescences were affected rising from 3.6 to 8.8 ($p \leq 0.05$) and from 5.1 to 16.3 shoots per 100 nodes ($p \leq 0.01$), respectively. PBZ significantly reduced the number of vegetative shoots per 100 nodes (0.8) compared to the control (3.8; $p \leq 0.05$).

Interestingly, the number of flowers per shoot of both leafy and leafless inflorescences was not significantly altered by GA_3 in comparison to the control, with 3.9 and 3.4 flowers per leafy inflorescence and 3.7 and 3.4 flowers per leafless inflorescence, respectively, whereas PBZ increased flower number significantly, up to 4.9 and 4.2 flowers per shoot for leafy and leafless inflorescences, respectively ($p \leq 0.05$). Neither GA_3 nor PBZ changed the number of leaves per shoot in any case, even that of vegetative shoots (data not shown).

The time course of the relative expression of *CiFT* in leaves throughout the study was significantly affected by GA_3 (**Fig. 1.10A**). Significant differences in mRNA transcripts between GA_3 treated trees and control trees were detected from 8 days after treatment (DAT) onward. The expression in control tree leaves increased progressively up to 32 DAT (mid-January), decreasing thereafter to almost the initial value (**Fig. 1.10A**). Gene expression in leaves of GA_3 treated trees paralleled that of control trees but was reduced by 16% on average, except for 80 DAT (late February) when no significant differences were found between control and treated trees (**Fig. 1.10A**). On the other hand, PBZ treatments trees significantly boosted the relative expression of *CiFT* in leaves (by 30% on average) from 8 DAT up to the end of February, which is the onset of bud sprouting. In this case, leaf gene expression also paralleled that of control trees, but with significantly higher values throughout the entire period studied. **Figure 1.10B** shows the time course of the relative expression of the *FLC-like* gene in leaves from control and GA_3 and PBZ treated trees. From early December to the onset of bud break, no differences in gene expression were found. Activity in leaves remained almost stationary between 0.70 and 1.03, regardless of the treatment.

Table 1.1 Effect of GA_3 (40 mg L^{-1}) and PBZ (2.000 mg L^{-1}) applied to entire tree during the floral bud inductive period (10 December) on bud sprouting and flowering of 'Salustiana' sweet orange trees.

	Control	GA_3	PBZ
Flowers	47.2 ± 3.1 b	13.5 ± 1.7 a	104.5 ± 9.2 c
Sprouted buds[a]	23.0 ± 1.9 b	13.8 ± 1.3 a	39.7 ± 2.8 c
Leafless shoots[b]			
Single flowered	3.6 ± 0.7 b	1.3 ± 0.5 a	8.8 ± 1.3 c
Inflorescence	5.1 ± 1.2 b	1.9 ± 0.2 a	16.3 ± 2.9 c
Leafy shoots			
Single flowered	0.8 ± 0.1 a	1.0 ± 0.2 a	0.9 ± 0.2 a
Inflorescence	5.6 ± 0.8 b	1.2 ± 0.3 a	5.7 ± 1.1 b
Vegetative shoots	3.8 ± 0.4 b	9.0 ± 0.5 c	0.8 ± 0.1 a
No. flowers inflorescence^{-1}			
Leafy inflorescence	3.9 ± 0.3 a	3.4 ± 0.5 a	4.9 ± 0.1 b
Leafless inflorescence	3.7 ± 0.1 a	3.4 ± 0.3 a	4.2 ± 0.1 b

Each value is the mean of six trees ± SE. Different letters in the same line indicate significant differences ($p \leq 0.05$). [a] Sprouted buds expressed as percent of total buds. [b] Number of shoots and flowers expressed per 100 nodes.

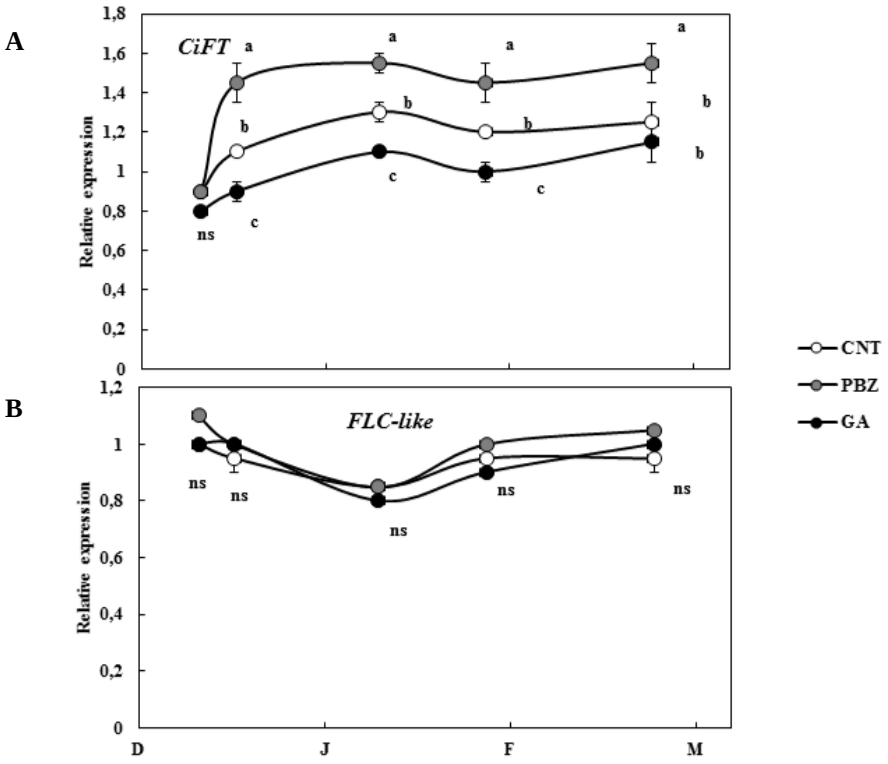


Figure 1.10 Effect of gibberellic acid (GA_3, 40 mg L^{-1}) and PBZ (2.000 mg L^{-1}) applied on December 10th on the time course of *CiFT* and *FLC-like* expression in the leaves of the 'Salustiana' sweet orange. Different letters for the same sampling date indicate significant differences ($p \leq 0.05$).

Control and GA_3-treated trees did not differ significantly in the relative expression of *CsLFY* in leaves, remaining almost constant between 0.66 and 1.21 throughout the study (**Fig. 1.11**). However, in PBZ-treated trees there was no treatment effect until late in January when mRNA transcripts in the leaf significantly increased 1.8-fold (1.94) compared to control (1.12). In spite of the subsequent decline, a significantly higher relative expression of *CsLFY* in comparison to control and GA_3-treated trees was recorded in PBZ-treated leaves at bud break. This effect coincided with the significant inhibition of *GA20ox1* activity, which was observed (**Fig. 1.11**).

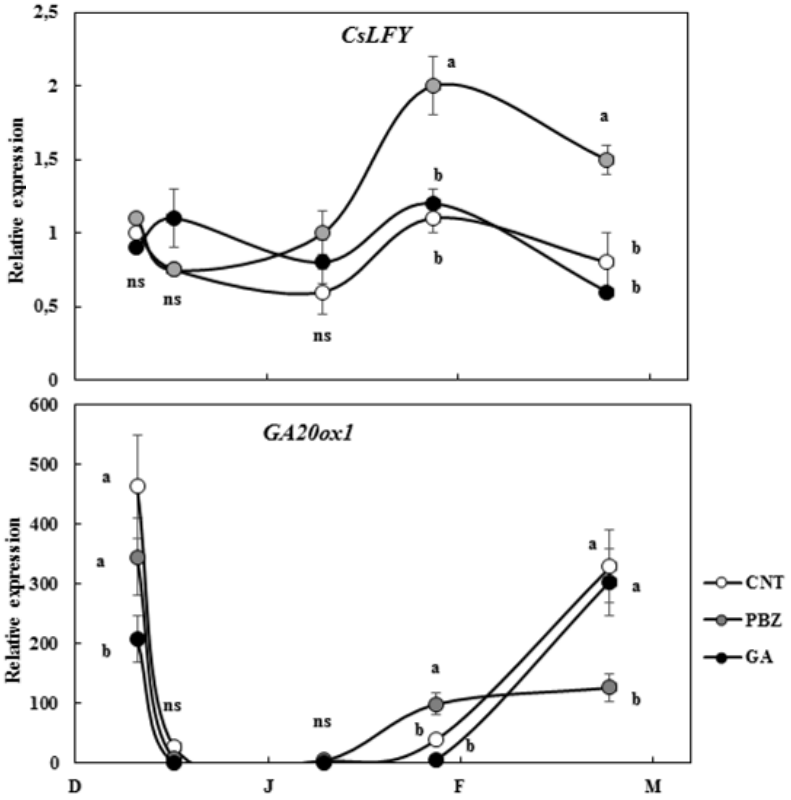


Figure 1.11 Effect of gibberellic acid (GA_3, 40 mg L^{-1}) and PBZ (2.000 mg L^{-1}) applied on December 10th on the time course of *CsLFY* and *GA20ox1* expression in the leaves of the 'Salustiana' sweet orange. Different letters for the same sampling date indicate significant differences ($p \leq 0.05$).

Results suggest that, under medium-yield conditions, treatments that modify GA metabolism can quantitatively modify flowering, i.e. the number of flowering shoots produced, by interfering in either flower induction genes (*FT*) or flower differentiation genes (*LFY*). The inhibitory flowering effect of GA has been previously related with the leaf and flower induction (Guardiola *et al.,* 1982) but also with the bud and flower differentiation (García-Luis *et al.,* 1986; Goldberg-Moller *et al.,* 2013).

ON and OFF trees

Figure 1.12 shows the influence of fruit load on flowering in the following spring for untreated and PBZ-treated trees. A significant inverse relationship was found between fruit load and flowering intensity in control and PBZ-treated trees of 'Salustiana' sweet orange (**A**) and 'Afourer' mandarin (**B**). Results also suggest that PBZ increased flowering only in low- and medium-yield trees and not in those with a heavy fruit yield, the threshold value being about 100–125 kg tree^{-1} for 'Salustiana' and 'Afourer'. For larger crop loads, no significant crop load – flowering intensity relationship was detected, whereas for lower crop loads a close relationship was observed. Thus, for instance, when PBZ was applied to both 'Salustiana' sweet orange and 'Afourer' mandarin trees bearing 50 kg tree^{-1}, the number of flowers per 100 nodes doubled from 33 to 64 and from 59 to 110, respectively; this effect was not achieved when PBZ was applied to trees with a crop load ≥125 kg tree^{-1} (**Fig. 1.12A and 1.12B**).

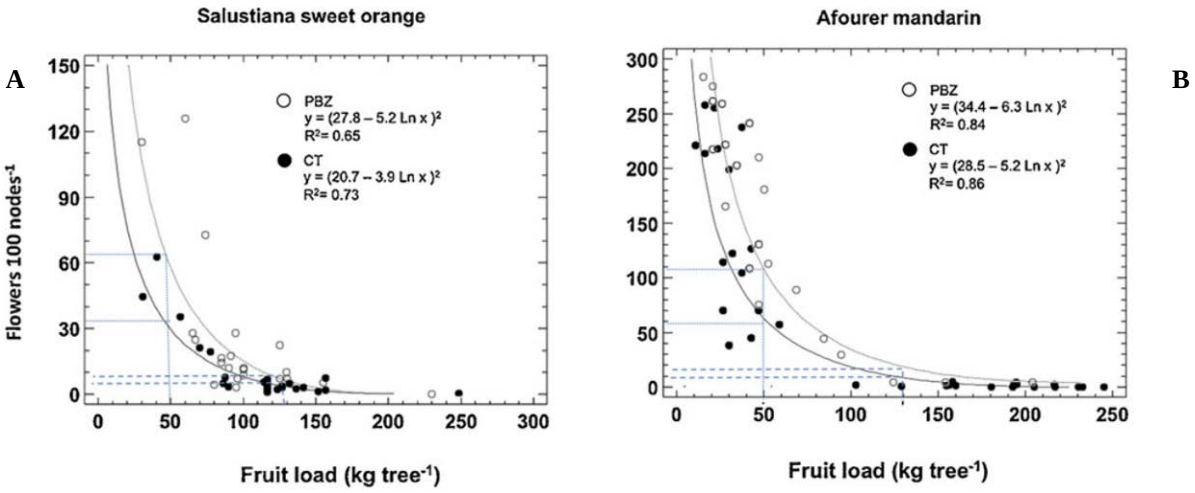


Figure 1.12 (A-B) Fruit load and flowering intensity relationship in 'Salustiana' sweet orange **(A)** and 'Afourer' mandarin **(B)** control and PBZ-treated trees (n = 23). PBZ (1 g tree^{-1}) was applied to the soil whereas GA_3 (50 mg l^{-1}) was applied as a foliar spray on November 25. Regressions [$y = (a + b^* \ln x)^2$] are significant at $p < 0.01$.

Furthermore, under ON-tree conditions, PBZ failed to promote flowering regardless of the dose applied, date or method of treatment or cultivar (**Fig. 1.13**). There was a significant increase in flowering intensity the following spring in OFF-trees of 'Salustiana' sweet orange and 'Afourer' mandarin receiving 1 g tree^{-1} or 10 g tree^{-1} to

the soil during the floral bud inductive period. However, ON-trees receiving the same doses flowered in proportions similar to untreated ON-trees, i.e. next to nothing (**Fig. 1.13A**). A similar response was obtained with 15 g tree^{-1} PBZ sprayed onto the canopies of ON- and OFF-trees of 'Moncada' mandarin (**Fig. 1.13A**). Advancing the treatment date of PBZ (1 g tree^{-1}) to earlier in the rest period (October) or delaying it until floral bud differentiation (February) did not counteract the effect of fruit load, nor did it produce the effect obtained when the same dose of PBZ was applied in the floral bud inductive period (November), regardless of the cultivar or method of treatment (**Fig. 1.13B**).

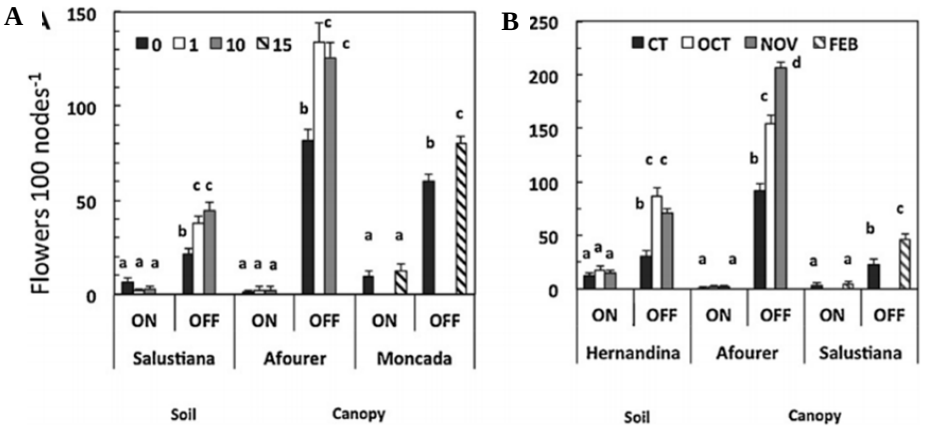


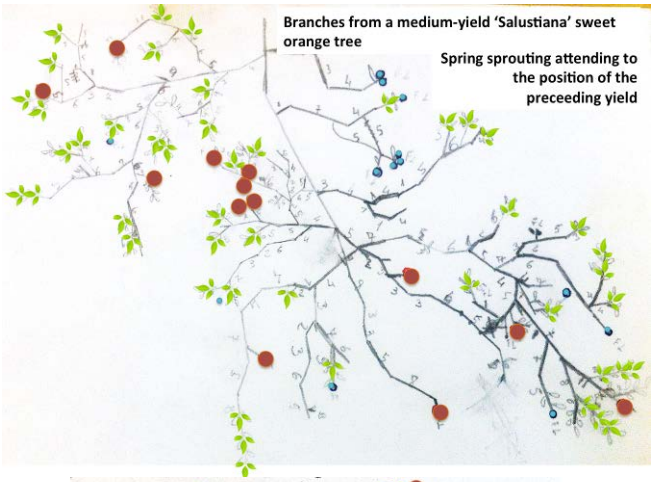
Figure 1.13 (A) Effect of PBZ dose (0, 1, 10, 15 g tree^{-1}) applied at floral bud inductive period (November 20-25) and **(B)** treatment date (October 10-15, November 20-25, February 20-24) (1 g tree^{-1}) (on flowering) in ON- and OFF-trees of 'Salustiana' sweet orange, 'Hernandina' clementine mandarin as well as 'Afourer' and 'Moncada' hybrid mandarins. PBZ was applied to the soil or as a foliar spray, and GA_3 was applied as a foliar spray. Data are means of 6-10 trees per cultivar. Standard errors are given as vertical bars. Different letters for the same cultivar indicate significant differences ($p \leq 0.05$). CT: control trees.

Treatments were also applied directly to the bud to further demonstrate its inability to flower in the presence of fruit. Thus, 2.5 μg PBZ applied locally to the buds of OFF-trees at the floral bud inductive period promoted flowering (113.5 flowers per 100 nodes) when compared to untreated buds (59.5 flowers per 100 nodes), but when PBZ was applied to the buds of ON-trees, it did not affect the number of flowers (7.6) compared to untreated ON-buds (7.8 flowers per 100 nodes).

1.4 Branch independence: the short-distance effect of the fruit. Fruit-shoot phloem transport interruption by peduncle girdling

Trees of 'Salustiana' sweet orange that bear 50 kg tree^{-1} on average are considered medium-yield trees (**Fig. 1.12**). Under these conditions, the tree is able to flower in spring. However, the inhibitory effect of the fruit is also evident if ON and OFF branches in the same tree are studied separately. For this research sprouting and flowering were evaluated in spring attending to the position of the preceding yield in branches of about 250-300 nodes, i.e, at least 2 years old. Results indicate a clear inverse relationship between fruit position and flowering, suggesting a short-distance effect of the fruit in flower induction inhibition (**Fig. 1.14**).

A



B

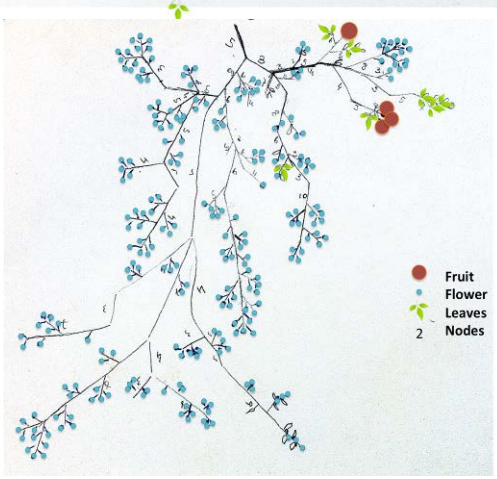


Figure 1.14 Schematic representation of sprouting and flowering in spring of two branches (250-300 nodes, 2 years old) from the same tree of 'Salustiana' sweet orange. Branches had 13 (**A**) and 4 (**B**) fruits and flowered very low and very high, respectively. An inverse relationship between fruit position and flowering is observed.

Therefore, in order to induce flowering in fruit bearing branches, an experiment was designed to interfere in this *short distance dominance*.

Spring branches with 8 nodes and a fruit in terminal position were girdled in the fruit peduncle at the end of stage two of fruit development, i.e., end of summer (August 25, 2014) (**Fig. 1.15A**). Ungirdled branches and OFF branches were selected for comparison. Girdling did not cause the abscission of the fruits, which remained on the branch until harvest. However, girdling significantly modified fruit development. At harvest, 7 months after girdling (March), girdled fruits were significantly smaller and their exocarps were greener than control fruits. The pulp changed color as did control fruit but the girdled fruit accumulated fewer TSS (total soluble solids) (**Table 1.2**).

Table 1.2 Characteristics of fruits from ON and Girdled shoots (7 months after girdling) in 'Afourer' hybrid mandarin. Each value is the mean of 10 fruits ± SE.

	Control	**Girdling**	**Significance**
Weight (g)	92.8±23.3	32.5±8.7	*
Diameter (mm)	57.5±4,8	43.2±2.9	*
Exocarp color			
a	28.8±1.1	-1.03±0.89	*
b	33.9±0.5	19.8±1.2	*
a/b	0.9±0.0	-0.1±0.1	*
Pulp color			
a	10.8±0.4	11.4±0.4	Ns
b	19.6±0.3	21.3±0.3	Ns
a/b	0.5±0.0	0.53±0.02	Ns
TSS (ºBrix)	16.4±0.5	8.5±0.2	*

Eleven days after girdling, the proximal buds sprouted in 15% of the girdled branches, and 22 days after girdling bud sprouting was achieved in 45% of the branches. In contrast, un-girdled branches (ON) did not sprout. Non-bearing branches (OFF) also sprouted naturally (fall flush) (**Fig. 1.15**).

Bud sprouting occurred in the 4 nodes closest to the girdle. From node #5 to node #8, buds did not sprout (**Fig. 1.16**). All the sprouted buds produced only vegetative shoots, which were longer in the girdled branches than in the OFF branches (**Fig. 1.17**).

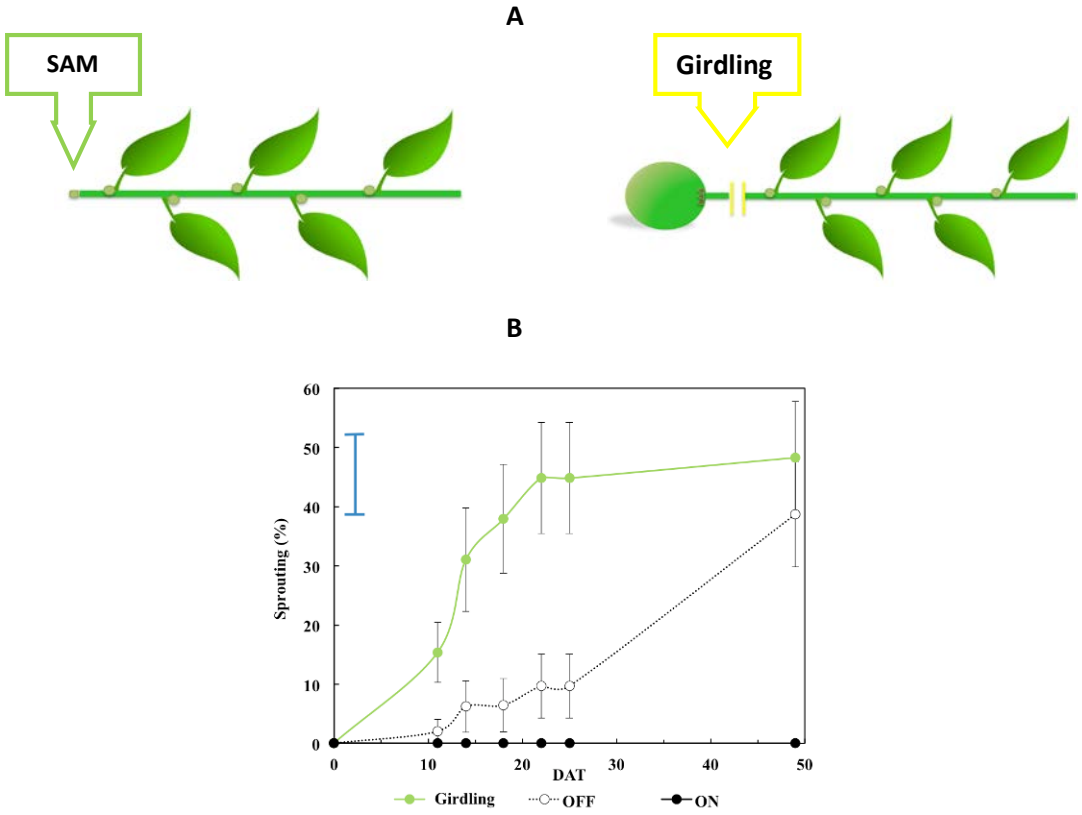


Figure 1.15 A. Representative figure of the type of shoots used for the experiment, vegetative shoots (OFF), single flowered leafy shoot (ON and Girdling). **B.** Evolution of the percentage of sprouting at the end of summer in single flowered leafy shoots (ON) and vegetative shoots (OFF) of 'Afourer' mandarin. Effect of girdling the peduncle of the fruit (Girdling). Each value is the mean of 50 shoots. Vertical bars represent the standard error. The blue bar shown on the left of the figure corresponds to LSD interval.

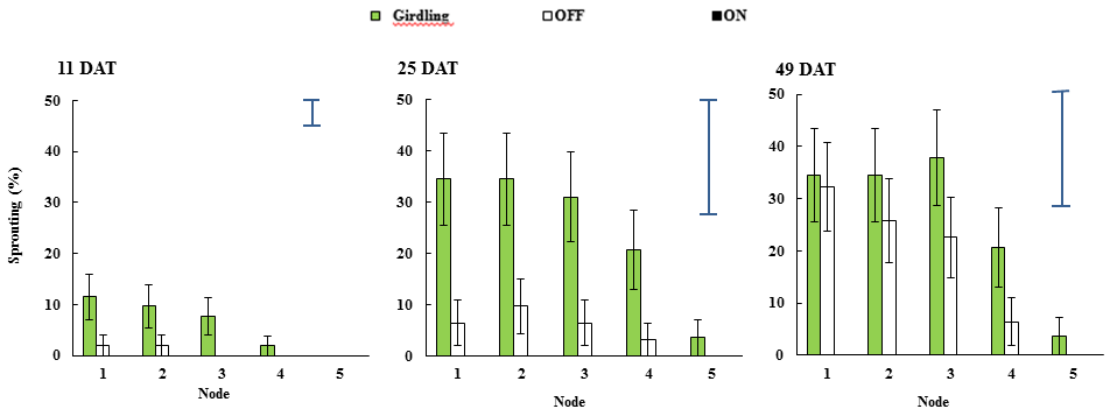


Figure 1.16 Distribution of sprouting at the end of summer along the nodes in single flowered leafy shoots (ON) and vegetative shoots of 'Afourer' mandarin. Effect of girdling the peduncle of the fruit (Girdling). Each value is the mean of 50 shoots. Node #1 corresponds to the node closest to the fruit/apex, while node #5 is the farthest. Vertical bars represent the standard error. The blue bar shown on the left of the figure corresponds to LSD interval.

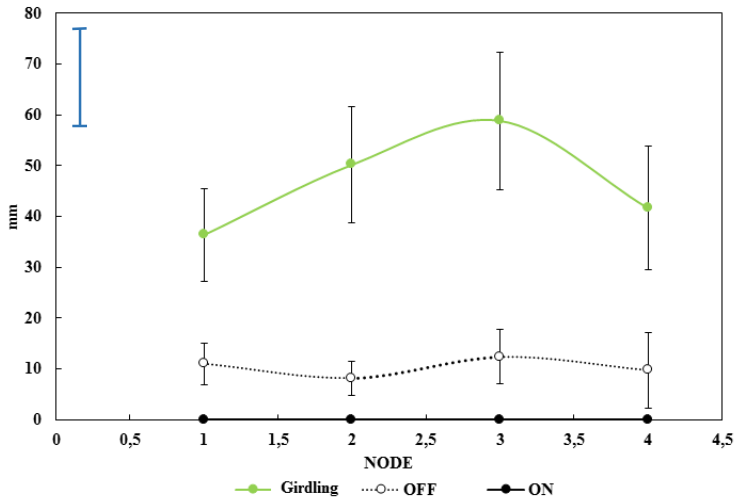


Figure 1.17 Length of the shoots at the end of summer, along the nodes in single flowered leafy shoots (ON) vegetative shoots (OFF) of 'Afourer' mandarin. Effect of girdling the peduncle of the fruit (Girdling). Each value is the mean of 50 shoots. Node #1 corresponds to node closest to the fruit/apex, while node #5 is the farthest. Vertical bars represent the standard error. The blue bar shown on the left of the figure corresponds to LSD interval.

Bud sprouting was related to changes in the hormonal balance in the nodes. In the un-girdled ON branches, the IAA content in the fruit was 7 ng g^{-1} in both the exocarp (ex) and the mesocarp+pulp (me+p) (**Fig. 1.18**). Further, the IAA content was significantly higher in the nodes than in the fruit. The third node showed the maximum IAA level (200 ng g^{1}). No GA_1 was found in the fruit while in the nodes GA_1 concentration was around 1 ng g^{-1} (**Fig. 1.18**). Girdling modified the hormonal balance; thus, it significantly reduced IAA concentration in the pulp (2 ng g^{-1}) and in the nodes (100 ng g^{-1} in the third node), and also *t*-Zeatin in the nodes compared to the ungirdled branch (**Fig. 1.18**). On the other hand, girdling significantly increased GA_1 in the nodes (3.4 ng g^{-1}). Other hormones (GA_4, ABA and JA) were not modified by the treatment (**Fig. 1.18**).

Nonetheless, the most outstanding result is that girdling of fruit peduncle triggered flowering the next spring while the ON branches did not flower. The girdled branches produced 45 flowers per branch, on average (**Fig. 1.19**). But flowers were only produced on the girdled branches that sprouted in September, that is, on buds from the fall shoots (**Fig. 1.19**). Girdled branches that did not sprout in September did not flower in March (**Fig. 1.19**). This result suggests that flowering in the fruiting branches was not directly due to girdling but to sprouting. On the other hand, all the meristems from OFF branches had the ability to flower (even without fall shoots). In this case, fall sprouting also increased flowering (**Fig. 1.19**).

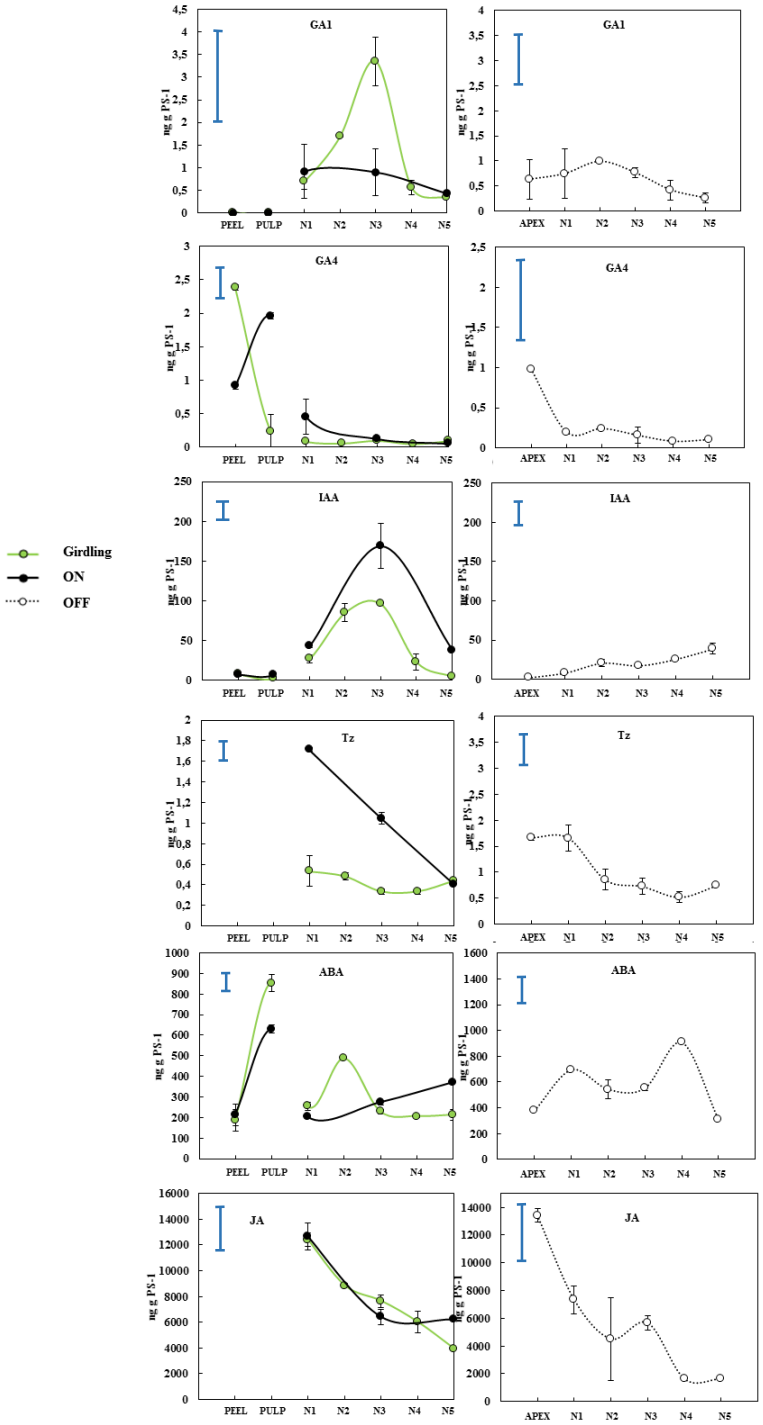


Figure 1.18 Effect of girdling on the endogenous GA_1, GA_4, IAA, TZ, ABA and JA content along the OFF, ON and Girdled shoots 11 days after girdling (August 25) in 'Afourer' hybrid mandarin. N1-N5: nodes next to the fruit; Girdling: of fruit peduncle. Data are means ± ES of 2 sets of 10 vegetative shoots/ leafy single flowered shoots/girdled leafy singleflowered shoots. The blue bar shown on the left of the figure corresponds to LSD interval.

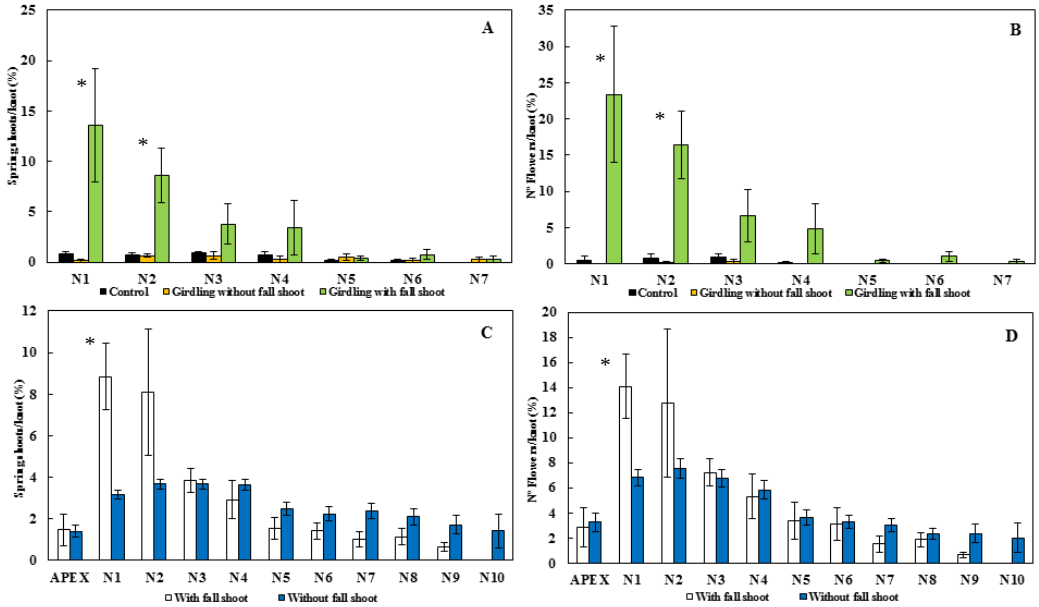


Figure 1.19 Girdling (August 2014) influence on sprouting and flowering in spring (March 2015). Experiment performed in the 'Afourer' hybrid mandarin. Number of spring shoots (**A** and **C**) and flowers (**B** and **D**) per node, in ON shoots (**A** and **B**), control and girdled, and OFF shoots (**C** and **D**); with fall shoot: this shoot sprouted in autumn and the spring sprouting in 2015 was analyzed on spring and autumn nodes of 2014; without fall shoot: spring sprouting in 2015 was analyzed on spring nodes of 2014. Data are means of 10 shoots. Standard errors are given as vertical bars. *: indicate significant differences ($p \leq 0.05$).

This important result suggests that meristems beside a fruit must be *restarted* to activate have the flowering ability. If this *renewed ability* is due to the development of new leaves, new buds or both then it is worthy of further examination. The lack of flowering in un-sprouted buds could be due to a *continuous inhibitory effect* even after the fruit reaches its final size and ripens, and not only from the fruit located in the terminal position but also from other fruits on the branch. To test this hypothesis, buds (nodes) from ON and OFF branches, similar to those from the girdling experiment, were excised and cultured *in vitro* (without the effect of the tree) before and after the flower induction period. Sprouting and flowering were evaluated.

1.5 *In vitro* culture of excised buds from ON and OFF branches

As derived in Materials and method, the experiment was conducted with the seedy mandarin 'Tardivo di Ciaculli', which is similar to 'Moncada' mandarin. Both are late ripening cultivars related to the Clementine mandarin species**.**

In spring, the OFF branches sprouted 22.5 shoots 100 nodes^{-1} while ON branches sprouted significantly fewer, 2 shoots 100 nodes^{-1}. OFF branches had the ability to flower (producing 10 flowers 100 nodes^{-1}) while ON branches did not (**Fig. 1.20**).

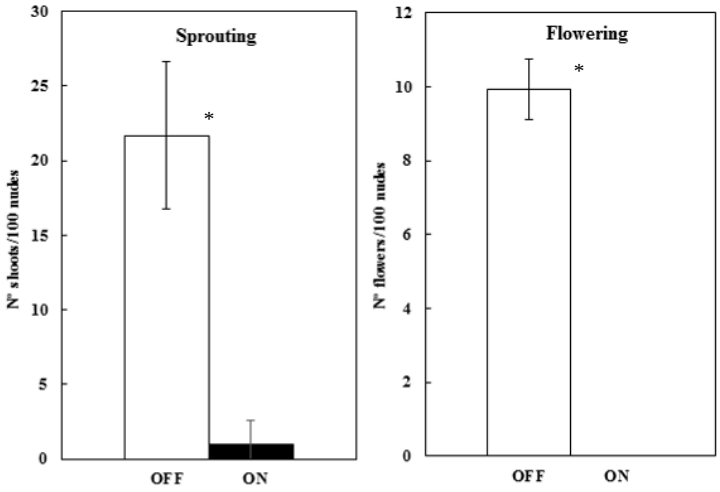


Figure 1.20 Number of shoots and flowers per 100 nodes of vegetative shoots (OFF) and leafy single flowered shoots (ON) of 'Mandarino Tardivo di Ciaculli' mandarin. Data are means ± ES of 30 shoots per treatment. *: indicate significant differences ($p \leq 0.05$).

Nodes from OFF and ON branches were cultured *in vitro* during (November) and after (February) the flower induction stage. At these dates, the fruit presented the characteristics described in the **Table 1.5**.

Table 1.5 Characteristics of fruits from leafy single flowered shoots in 'Mandarino Tardivo di Ciaculli' mandarin. Each value is the mean of 10 fruits ± SE.

	November	February
Diameter (mm)	25.1 ± 0.7	39.0 ± 0.8
Exocarp color		
a	-14.5 ± 0.3	30.1 ± 0.7
b	22.8 ± 0.8	32.0 ± 0.7
a/b	-0.65 ± 0.04	0.93± 0.05
Number of seeds	7.2 ± 0.5	n.d

In November, bud sprouting was triggered *in vitro* during the first 2 weeks of the experiment (**Fig. 1.21A and B**). OFF-nodes sprouted up to 30% whereas only 5% of ON-nodes sprouted. To stimulate bud sprouting, zeatin was added to the growing medium, which was based on carbohydrates, macronutrients and micronutrients. Zeatin increased bud sprouting significantly in both OFF-nodes (52%) and ON-nodes (12%) (**Fig. 1.21B**). No flowering was achieved *in vitro*. Sprouted buds only produced vegetative shoots in both OFF and ON nodes (**Fig. 1.22**).

In order to ensure that the flowering signal (FT) had reached the bud due to winter low temperature, at least in OFF branches, a second set of nodes were harvested and cultured *in vitro* in February. At this time, bud sprouting was also triggered during the first 2 weeks, but a higher percentage of sprouted buds was found compared to November, particularly for that of ON-nodes (**Fig. 1.21**). Thus, 15 days after culture, ON-buds sprouted up to 33% and 55% in medium without and with zeatin, respectively, (6% and 12% in November) (**Fig. 1.21B and C**). Sixty days after culture the ON-bud percentage of sprouting reached 51% and 71% in medium without and with zeatin, respectively (**Fig. 1.21B and C**). In February, differences in bud sprouting between ON and OFF buds were significantly narrowed compared to November.

Results suggest that OFF-buds are able to sprout when exogenous factors are favorable whereas ON-buds cannot sprout until the 'fruit inhibitory signal' disappears (as it seemed to occur in February).

It is worth nothing that flowering was inhibited in both ON and OFF buds cultured *in vitro*. That is, ON buds could not flower as they did on the tree, and OFF buds also lost

their ability to differentiate flowers when separated from the tree, even after the flower induction period.

The fact that a meristem beside a fruit needs to be *restarted* to gain the flowering ability, and that the application of PBZ does not induce flowering under heavy ON-tree conditions, suggests that flowering is the result of complex interactions at the metabolic and molecular level, involving multiple endogenous mechanisms of regulation different from hormone concentration and transport. It is still unclear which main metabolic routes and biological processes are up-regulated and down-regulated in trees with contrasting ability to flower due to the presence of fruit. Therefore, to attempt to clarify these questions, a proteomic study of leaves and buds from ON and OFF trees was conducted during the flower induction period.

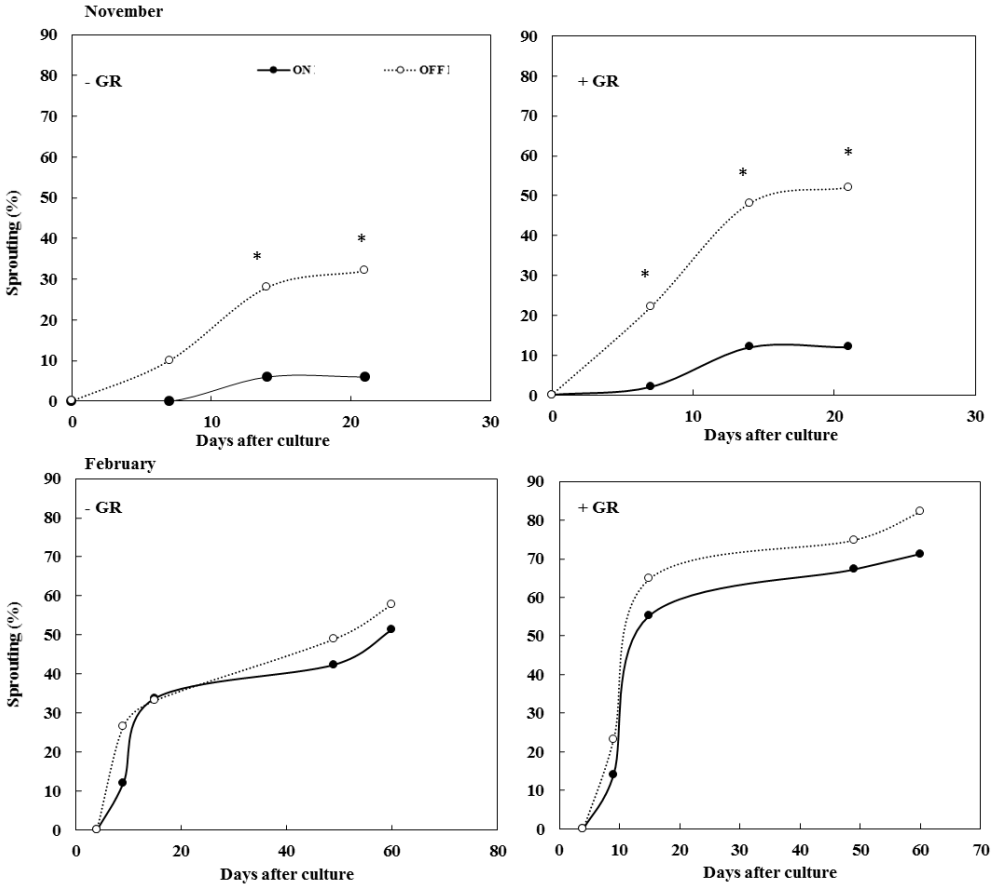


Figure 1.21 Effect of the fruit and the medium composition (-GR/+GR) on the sprouting *in vitro* of microshoots with one bud in 'Mandarino Tardivo di Ciaculli'. Each value is the mean of 10 dishes with 5 microshoots. GR: growth regulator (zeatin (1 mg/ml)). *: indicate significant differences ($p \leq 0.05$).

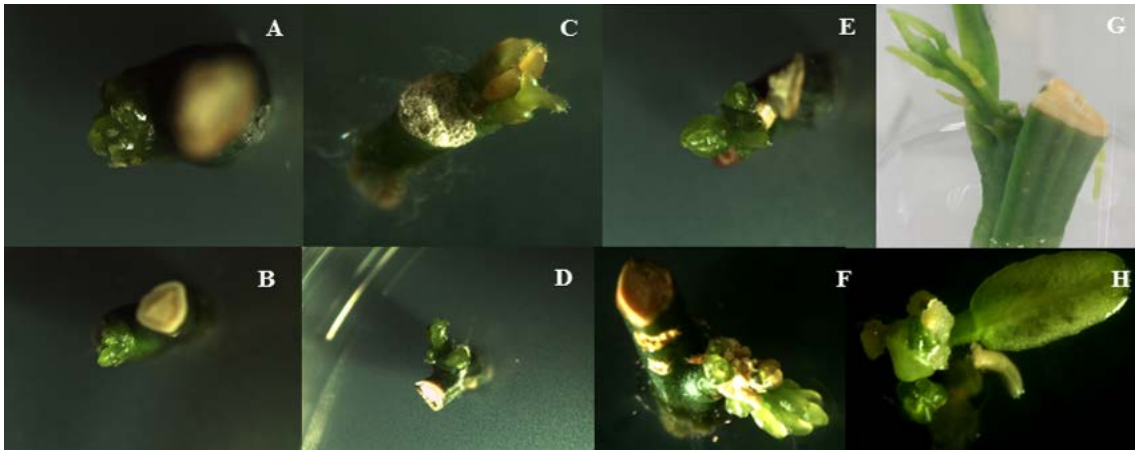


Figure 1.22 Growth of microshoots in the culture medium. Photographs taken after 7 (**A**), 14 (**B**) 21 (**C**), 28 (**D**), 35 (**E**), 42 (**F**), 56 (**G**), 60 (**H**) days of culture. OFF microshoots.

Section 2. Proteomic analysis of "Moncada" mandarin leaves and buds with contrasting fruit load

2.1 Comparative proteome analysis

The protein spots were identified that were up- and down-regulated in OFF trees comparing with ON trees. Therefore, leaf and bud samples from OFF and ON trees were analyzed by 2D DIGE gel, two-dimensional difference gel electrophoresis. Gels were of high quality with reproducible protein patterns among replicates of the same samples (**Fig. 2.1**).

Approximately 1436 and 1162 spots in gel images from samples were resolved in leaves and buds, respectively. To assess global differences in the expression levels between OFF and ON samples, gels were compared and quantified using the DeCyder Differential Analysis Software. Among the total proteins, 176 spots showed a significant quantitative differential accumulation (t-test < 0.05) between ON and OFF samples in leaves, while there were 350 spots in buds. 110 spots in leaves and 192 in buds were confirmed with a good match and a sufficient volume for subsequent identification by mass spectrometry. To reliably determine quantitative changes in protein expression and therefore overcome error imposed by technical and biological variations, proteins were identified as up-regulated in OFF or ON samples if they were found to have an average expression level at least 1.10 higher than the other ON or OFF samples, respectively. Among the 110 proteins in leaves, 43 had increased expression in the OFF samples compared to ON samples (Av ratio +), while 67 showed a decreased expression in the OFF samples (Av ratio -). In buds, 97 displayed an increased expression in the OFF samples as compared to the ON samples (Av ratio +), while 95 exhibited a decreased expression in the OFF samples (Av ratio -).

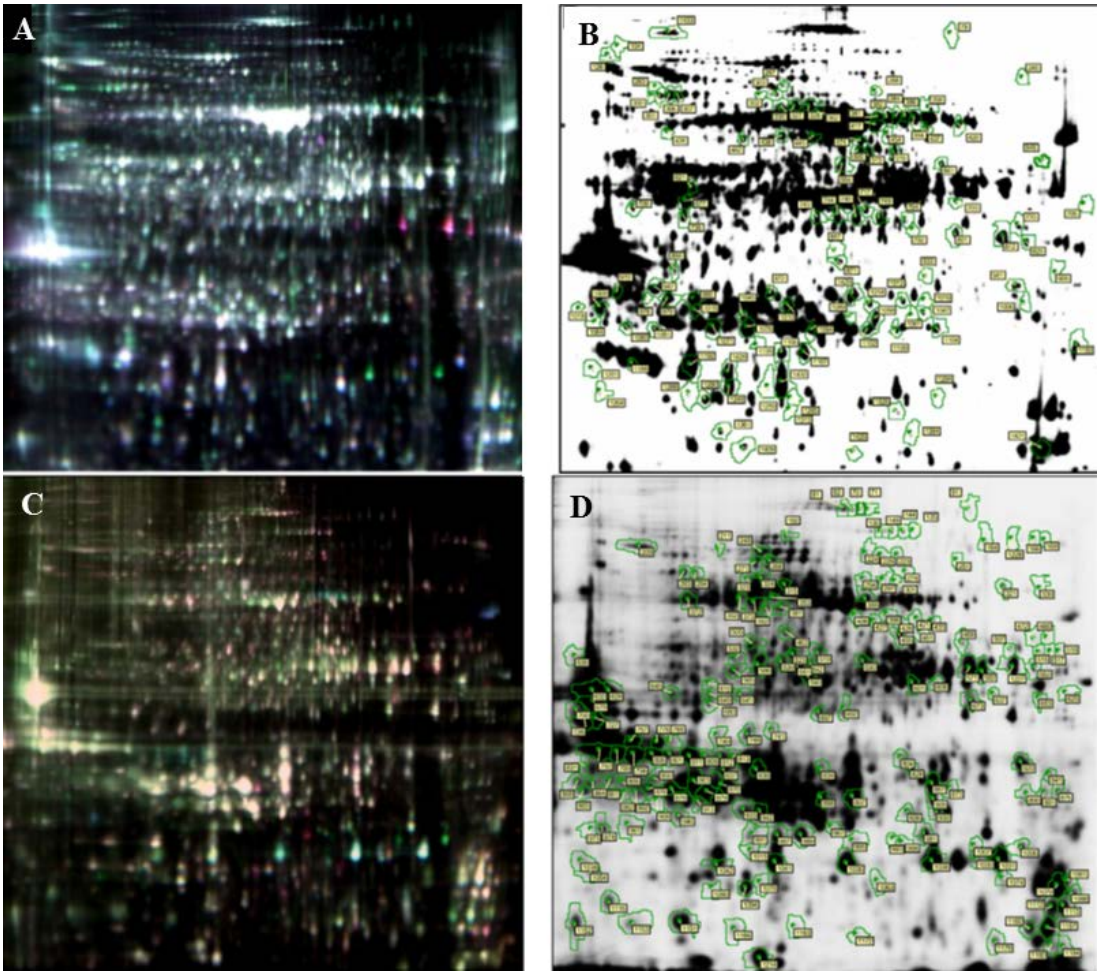


Figure 2.1 Representative 2D DIGE gel of proteins extracted from 'Moncada' mandarin leaves (**A-B**) and buds (**C-D**). Equal amounts (50µg) of ON sample (Cy5, red), OFF sample (Cy3, green) and internal standard (Cy2, blue) were loaded in the same gel. (**A-C**) Proteins up-expressed in the OFF appear in green, those down-expressed in OFF appear in red and proteins unaffected appear in yellow. (**B-D**) Proteins selected for the analysis by mass spectrometry. Spot numbers correspond to those indicated in **Tables 2.1** and **2.2.**

2.2 Identification of differentially expressed proteins

The 110 proteins in leaves and 192 in buds were manually excised with a good match from a preparative 2DE gel to further identify 90 leaves and 88 buds by MALDI-MS analysis, and the other 20 leaves and 104 bud proteins by LC-MS/MS analysis. **Table 2.1** and **Table 2.2** provide the spot number, the function of each protein together with the putative protein name, the accession code, the organism based on the protein identified, the homologue in *C. clementina* established by the database in

www.phytozome.net (Phytozome v9.1), the homologue in *Arabidopsis thaliana* established by the database in www.arabidopsis.orgs, the values for theoretical and experimental pI and molecular mass, the expression ratio and p-value, and the MASCOT score together with the sequence coverage and peptides matched.

Table 2.1 List of identified proteins in leaves.

Spot	Function/Protein	Accession No.	Species	Homolog in *Citrus clementina*	Homolog in *Arabidopsis thaliana*	Mol. Mass (kDa) Theo /Exp	p*I* Theo /Exp	ratio	*p*-value	Score/sequence coverage (%)/ peptides matched
	(i) Related to primary metabolism Photosynthesis and carbohydrate metabolism									
238	NADP-dependent malic enzyme	12712UC37CL5763 Contig3	*Vitis vinifera*	clementine0.9_005680m	AT1G79750.1	75.79/ 81.50	8.58/7.45	-1.27	0.050	110/20/15
267	ATP-binding cassette transporter [a]	98077UC452943	*Pteris vittata*	clementine0.9_009053m	AT5G60790.1	88.65/80.00	8.62/7.60	-1.22	0.033	233/6/5
288	Putative t-complex protein 1 theta chain	144062UC3813154	*Magnoliophyta*	clementine0.9_006345m	AT3G11830.1	79.87/79.50	8.55/8.70	1.23	0.025	82/27/17
300	RuBisCO large subunit-binding protein subunit beta [b]	13227UC37CL6180 Contig2	*Brassicaceae*	clementine0.9_005361m	AT5G56500.2	74.41/79.00	6.73/6.10	1.83	0.034	172/28/17
301	RuBisCO large subunit-binding protein subunit beta [b]	13227UC37CL6180 Contig2	*Brassicaceae*	clementine0.9_005361m	AT5G56500.2	74.41/79.50	6.73/6.15	1.81	0.025	174/26/15
302	Phosphoglucomutase [a, b]	46090UC37041305	*Brassicaceae (Arabidopsis thaliana)*	clementine0.9_004855m	AT5G51820.1	30.55/78.00	7.86/7.50	-1.19	0.042	319/31/8
303	3,4-dihydroxy-2-butanone kinase [a]	15551UC37CL8203 Contig1	*Solanum licopersicum*	clementine0.9_005619m	AT3G17770.1	67.21/79.50	6.46/7.40	-1.23	0.012	607/15/11
306	RuBisCO large subunit-binding protein subunit beta [a,b]	136270UC385359	*Brassicaceae*	clementine0.9_005361m	AT1G55490.2	80.16/80.00	8.93/6.20	1.70	0.025	1463/33/26
307	RuBisCO large subunit-binding protein subunit beta [b]	13227UC37CL6180 Contig2	*Brassicaceae*	clementine0.9_005361m	AT5G56500.2	74.41/80.00	6.73/6.25	1.66	0.025	307/34/21
330	Ribulose-1.5-bisphosphate carboxylase/oxygenase large subunit	gi|21634083	*Dichondra brachypoda*	clementine0.9_008884m	ATCG00490.1	51.69/70.00	6.09/7.60	1.19	0.025	104/26/14
362	Ribulose-1.5-biphosphate-carboxylase	gi|6634076	*Citrus paradisi*	clementine0.9_008884m	ATCG00490.1	52.03/70.00	6.19/8.00	-1.15	0.046	185/31/18
417	Ribulose bisphosphate carboxylase large chain	RBL_CITSI	*Citrus sinensis*	clementine0.9_008884m	ATCG00490.1	53.00/68.50	6.29/8.80	-1.28	0.025	339/38/24
420	Nadp-dependent glyceraldehyde-3 phosphate dehydrogenase	99276UC454142	*Medicago truncatula*	clementine0.9_008326m	AT2G24270.4	63.30/68.50	8.73/9.45	1.23	0.029	204/44/14
437	Nadp-dependent glyceraldehyde-3-phosphate dehydrogenase	14638UC37CL7392 Contig2	*Medicago truncatula*	clementine0.9_008326m	AT2G24270.4	61.03/65.50	8.76/9.00	1.20	0.021	177/21/11
438	AlaT1	14418UC37CL7192 Contig2	*Vitis vinifera*	clementine0.9_008926m	AT1G70580.4	67.99/62.00	8.56/7.55	1.17	0.029	112/21/14
445	Ribulose-1.5-bisphosphate carboxylase/oxygenase large subunit	gi|296277499	*Atalantia spinosa*	clementine0.9_008884m	ATCG00490.1	48.05/61.50	6.30/5.85	-1.16	0.022	235/32/20
462	ADP-glucose pyrophosphorylase small subunit	gi|111660950	*Citrus sinensis*	clementine0.9_007503m	AT5G48300.1	57.33/62.00	6.73/7.05	1.24	0.007	235/33/16
554	Alcohol dehydrogenase	12873UC37CL5895 Contig1	*Citrus paradisi*	clementine0.9_036084m	AT1G77120.1	57.84/56.50	8.30/8.20	-1.22	0.018	77/18/8
743	Putative cinnamoyl-CoA reductase	2277UC37CL154 Contig7	*Arabidopsis thaliana*	clementine0.9_027444m	AT2G02400.1	39.14/48.00	8.38/7.90	1.20	0.033	155/24/7
744	Putative cinnamoyl-CoA reductase [a]	2277UC37CL154 Contig7	*Arabidopsis thaliana*	clementine0.9_027444m	AT2G02400.1	39.14/49.00	8.38/8.00	1.30	0.023	105/8/3
748	Putative cinnamoyl-CoA reductase	2277UC37CL154 Contig7	*Arabidopsis thaliana*	clementine0.9_027444m	AT2G02400.1	39.14/49.00	8.38/8.40	1.45	0.019	209/34/11
847	Carbonic anhydrase [a]	1035UC37CL1 Contig1036	*Populus tremula x Populus tremuloides*	clementine0.9_015268m	AT3G01500.2	43.81/46.00	7.95/8.00	1.22	0.033	247/19/6
894	Transketolase. chloroplastic	TKTC_MAIZE	*Zea mays*	clementine0.9_003238m	AT3G60750.1	73.35/45.00	5.47/6.25	-1.40	0.030	59/7/6

970	Putative ribose 5-phosphate isomerase [a]	2390UC37CL176 Contig5	*Arabidopsis thaliana*	clementine0.9_018380m	AT3G04790.1	40.34/43.00	9.22/5.60	-1.18	0.044	349/21/11
1016	Miraculin-like protein 1 [a]	902UC37CL1 Contig903	*Citrus jambhiri*	clementine0.9_020626m	AT1G17860.1	35.05/42.50	9.50/9.00	1.74	0.016	422/29/13
1049	Miraculin-like protein 1 [a]	184UC37CL1 Contig184	*Citrus jambhiri*	clementine0.9_020626m	AT1G17860.1	35.58/41.50	9.38/8.25	1.75	0.015	454/28/13
	Krebs cycle									
500	Citrate synthase	CISY_CITMA	*Citrus maxima*	clementine0.9_009277m	AT2G44350.1	52.43/59.00	6.90/8.45	-1.27	0.027	212/26/14
515	NADP-isocitrate dehydrogenase	gi|5764653	*Citrus limon*	clementine0.9_006693m	AT1G65930.1	46.82/ 58.00	6.49/8.50	-1.24	0.025	79/23/10
516	NADP-isocitrate dehydrogenase	gi|5764653	*Citrus limon*	clementine0.9_006693m	AT1G65930.1	46.82/58.00	6.49/8.70	-1.22	0.011	164/31/15
764	Malate dehydrogenase, glyoxysomal precursor	143397UC3812489	*Citrullus lanatus*	clementine0.9_011590m	AT2G22780.1	49.76/48.50	9.43/8.80	1.25	0.021	95/17/6
	Pentose phosphate pathway									
454	6-phosphogluconate dehydrogenase	3692UC37CL541 Contig4	*Arabidopsis thaliana*	clementine0.9_008389m	AT1G64190.1	67.85/62.00	8.52/8.70	1.19	0.025	99/18/10
	Related to nutrient reservoir activity									
327	Granule-bound starch synthase Ib precursor	12888UC37CL5907 Contig1	*Phaseolus vulgaris*	clementine0.9_005261m	AT1G32900.1	82.61/74.00	8.87/7.75	1.40	0.012	221/25/16
328	Granule-bound starch synthase Ib precursor	12888UC37CL5907 Contig1	*Phaseolus vulgaris*	clementine0.9_005261m	AT1G32900.1	82.61/74.00	8.87/7.80	1.56	0.005	107/21/14
	(ii) Oxidoreductase activity									
79	Putative monocopper oxidase precursor	100961UC455827	*Arabidopsis thaliana*	clementine0.9_034025m	AT4G12420.2	83.18/89.0	9.12/9.30	2.94	0.005	128/23/16
381	Catalase	11460UC37CL4806 Contig1	*Prunus persica*	clementine0.9_008455m	AT4G35090.1	67.93/69.50	8.86/8.70	-1.26	0.011	362/28/18
382	Catalase	147681UC3816774	*Prunus persica*	clementine0.9_008455m	AT4G35090.1	68.54/69.00	8.99/8.75	-1.40	0.015	345/36/27
384	Catalase	11460UC37CL4806 Contig1	*Prunus persica*	clementine0.9_008455m	AT4G35090.1	67.93/69.00	8.86/8.80	-1.40	0.011	385/33/25
386	Catalase	11460UC37CL4806 Contig1	*Prunus persica*	clementine0.9_008455m	AT4G35090.1	67.93/69.00	8.86/8.85	-1.37	0.017	375/39/25
394	Catalase	11460UC37CL4806 Contig1	*Prunus persica*	clementine0.9_008455m	AT4G35090.1	67.93/69.00	8.86/8.90	-1.15	0.048	309/28/20
474	Monodehydroascorbate reductase	4200UC37CL707 Contig5	*Solanum lycopersicum*	clementine0.9_008242m	AT1G63940.2	60.85/63.00	8.77/8.50	-1.26	0.025	231/35/20
1135	Fe-Superoxide dismutase [a]	1143UC37CL6 Contig5	*Lotus japonicus*	clementine0.9_018576m	AT5G51100.1	41.15/37.50	8.86/7.35	-1.43	0.011	352/17/8
	(iii) Stress responses									
717	clone C31705D02	gi|218832267	*Citrus clementina*	clementine0.9_014588m	AT3G03080.1	27.31/49.50	6.92/ 8.40	-1.24	0.010	187/37/10
1249	Stress-related protein [a]	96234UC451100	*Citrus sinensis*	clementine0.9_023848m	AT5G45860.1	26.39/30.00	8.87/6.95	-1.81	0.012	489/35/12
1259	Stress-related protein [a]	12135UC37CL5321 Contig1	*Citrus sinensis*	clementine0.9_023848m	AT5G45860.1	27.07/30.50	9.34/7.45	-1.39	0.039	305/19/5
	(iv) Signal transduction									
736	Putative plastidic cysteine synthase 1	105791UC4510657	*Oryza sativa*	clementine0.9_013305m	AT2G43750.2	49.14/49.00	9.00/6.70	-1.17	0.036	222/44/22
	(v) Protein synthesis and degradation									
104	Protein disulphide isomerase [a]	4082UC37CL670 Contig2	*Elaeis guineensis*	clementine0.9_031693m	AT5G60640.1	36.28/88.00	5.38/5.40	1.30	0.042	534/31/12
126	Protein disulphide isomerase [a]	41019UC37AZ	*Elaeis guineensis*	clementine0.9_031693m	AT5G60640.1	32.97/86.50	5.35/5.30	1.24	0.036	413/28/11
358	Chaperonin subunit putative	101818UC456684	*Arabidopsis thaliana*	clementine0.9_007038m	AT3G18190.1	33.30/72.50	5.91/9.05	1.27	0.033	81/31/9
933	Tudor; Staphylococcus nuclease subtype [a]	6763UC37CL1850 Contig2	*Medicago truncatula*	clementine0.9_001246m	AT5G07350.2	39.58/43.50	6.16/9.00	1.38	0.023	196/7/4

1018	Cysteine proteinase-like protein	8069UC37CL2571 Contig1	*Ipomoea batatas*	clementine0.9_022372m	AT3G49340.1	41.17/42.00	5.67/5.20	-1.57	0.016	78/19/7
1264	Adenylate isopentenyltransferase 8, chloroplastic	IPT8_ARATH	*Arabidopsis thaliana*	clementine0.9_014843m	AT3G19160.1	37.58/30.00	8.75/5.40	1.26	0.016	61/45/15
	(vi) Expansins									
871	Xyloglucan endotransglycosylase	98350UC453216	*Gossypium hirsutum*	clementine0.9_017721m	AT5G65730.1	47.70/45.00	7.90/8.05	1.63	0.008	132/22/9
	(vii) Other proteins									
263	T13D8.29 protein [a]	31101UC37	*Arabidopsis thaliana*	clementine0.9_006161m	AT1G60420.1	25.79/81.00	4.95/6.10	1.30	0.007	407/26/9
428	Tubulin alpha-1 chain	4507UC37CL828 Contig2	*Arabidopsis thaliana*	clementine0.9_030036m	AT5G19780.1	64.11/67.00	5.64/5.90	-1.21	0.021	80/19/11
545	Putative pectinesterase [a]	19469UC37CL12030 Contig1	*Oryza sativa*	clementine0.9_008076m	AT4G33230.1	34.41/57.00	9.18/10.25	1.65	0.011	214/13/4
561	clone C05136B10	gi|63065066	*Citrus sinensis*	clementine0.9_009673m	AT1G30580.1	27.71/56.00	8.96/9.20	-1.14	0.030	234/70/19
621	clone IC0AAA48BC11	gi|218838992	*Citrus clementina*	clementine0.9_010996m	AT2G20420.1	31.93/54.00	5.91/6.65	-1.24	0.025	126/42/13
677	clone STG2_1309 5'	gi|209935988	*Citrus unshiu*	clementine0.9_011770m	AT5G50850.1	24.53/50.00	5.21/6.70	-1.10	0.042	90/34/8
703	clone UCRPT02-33B05-D10-1-6.b	gi|57873050	*Citrus trifoliata*	clementine0.9_020100m	AT2G38380.1	31.89/50.50	9.32/9.50	1.34	0.008	95/29/10
706	clone IC0AAA46BA07	gi|218790269	*Citrus clementina*	clementine0.9_014847m	AT1G71695.1	31.79/50.50	8.93/10.80	-2.51	0.005	133/35/8
709	Beta-1,3-glucanase class III [a]	13154UC37CL6125 Contig2	*Citrus clementina*	clementine0.9_014533m	AT3G57260.1	43.33/50.00	5.68/5.80	-1.29	0.048	412/19/9
749	IC0AAA37AD07RM1 CitNFL	gi|110857582	*Citrus clementina*	clementine0.9_020100m	AT2G02400.1	47.64/49.00	9.09/8.65	1.46	0.014	171/20/10
770	PT11-C9-005-009-C08-CT.F	gi|188225533	*Poncirus trifoliata*	clementine0.9_011590m	AT2G22780.1	29.28/48.00	9.38/10.00	1.60	0.027	108/43/9
792	clone IC0AAA10DG03	gi|218812293	*Citrus clementina*	clementine0.9_016626m	AT4G39230.1	34.13/47.50	9.61/9.00	-2.33	0.011	80/34/9
801	clone KN0AAH3CD04	gi|218820391	*Citrus clementina*	clementine0.9_016626m	AT4G39230.1	29.06/47.00	9.51/9.50	-2.78	0.012	358/50/14
812	clone IC0AAA10DG03	gi|218812293	*Citrus clementina*	clementine0.9_016626m	AT4G39230.1	34.13/47.00	9.61/9.90	-2.86	0.005	417/56/18
825	clone IC0AAA10DG03	gi|218812293	*Citrus clementina*	clementine0.9_016626m	AT4G39230.1	34.13/46.50	9.61/10.10	-3.22	0.005	114/33/9
909	KN0AAP9YD08FM1 Fruit-TF	gi|110851549	*Citrus clementina*	clementine0.9_021318m	AT1G09560.1	30.66/44.00	8.78/10.40	-1.64	0.025	138/14/5
941	clone UCRCS04_ 030_T3_C11	gi|55936798	*Citrus sinensis*	clementine0.9_006827m	AT5G66140.1	27.71/43.00	8.69/9.90	1.20	0.042	155/29/9
947	DC884625 ANT	gi|209925742	*Citrus unshiu*	clementine0.9_019708m	AT3G48420.1	28.53/43.00	6.73/6.10	-1.28	0.017	96/14/3
972	clone A1650001_IIF_C01 5'	gi|45451762	*Citrus sinensis*	clementine0.9_019618m	AT3G09640.2	32.25/42.50	6.19/6.65	-1.62	0.044	175/43/14
978	clone UCRCS08-3E10-J19-1-5.g	gi|56588623	*Citrus sinensis*	clementine0.9_012723m	AT1G49970.1	27.09/42.00	5.40/5.95	-1.18	0.030	94/65/14
979	clone C05808B11	gi|63065913	*Citrus sinensis x C. trifoliata*	clementine0.9_020467m	AT5G02790.1	27.01/42.50	5.58/6.20	-1.27	0.025	159/57/12
1006	IC0AAA29DA06RM1 CitNFL	gi|110851709	*Citrus clementina*	clementine0.9_003575m	AT1G21680.1	37.05/42.00	5.63/10.10	-1.75	0.023	123/28/7
1009	clone VPE-39_H09 5'	gi|7597421	*Citrus sinensis*	clementine0.9_020761m	AT1G17100.1	26.39/40.50	5.17/5.35	-1.61	0.016	86/31/5
1010	clone C31102C11	gi|218825114	*Citrus reshni*	clementine0.9_018172m	AT2G32520.1	24.79/42.50	8.59/7.60	-1.24	0.012	107/38/7
1012	UCRCR01_06K12_f	gi|38032410	*Citrus reticulata*	clementine0.9_014505m	AT4G09010.1	20.38/ 42.00	6.34/8.75	-1.20	0.026	91/49/8
1014	KN0AAL1BE04FM2 KCl-Salt1	gi|110886813	*Citrus reshni*	clementine0.9_035067m	AT3G22110.1	39.67/ 42.00	6.52/8.30	1.14	0.043	134/19/6
1015	Hypothetical protein At2g31670	142854UC3811946	*Arabidopsis thaliana*	clementine0.9_019220m	AT2G31670.1	30.98/ 42.00	8.88/6.7	-1.34	0.018	86/42/10
1045	CR05-C1-100-016-F12-CT.F	gi|188356206	*Citrus reticulata*	clementine0.9_000163m	AT3G01500.2	32.88/ 41.50	8.86/7.05	-1.40	0.036	74/39/9
1047	PT11-C1-900-008-E04-CT.F	gi|188446025	*Poncirus trifoliata*	clementine0.9_016969m	AT3G26340.1	32.80/ 42.00	8.47/9.00	1.24	0.017	119/33/12
1058	At1g76020	23150UC37CL15808 Contig1	*Arabidopsis thaliana*	clementine0.9_034879m	AT1G76020.1	27.52/41.50	10.19/8.70	-1.49	0.007	208/26/7
1061	Chitinase CHI1 [a]	14249UC37CL7048 Contig1	*Citrus sinensis*	clementine0.9_020680m	AT3G54420.1	41.25/41.00	8.42/8.95	-1.48	0.015	269/20/6
1064	clone UCRCS09-27B08-D16-1-5.g	gi|56533662	*Citrus sinensis*	clementine0.9_027803m	AT3G14290.1	31.80/40.50	4.75/5.30	-1.27	0.003	123/34/12
1071	CS00-C1-650-010-H07-CT.F	gi|188298712	*Citrus sinensis*	clementine0.9_015268m	AT3G01500.2	31.80/39.00	8.97/6.85	-1.22	0.025	123/53/14
1076	CS00-C1-650-010-H07-CT.F	gi|188298712	*Citrus sinensis*	clementine0.9_015268m	AT3G01500.2	30.96/39.50	8.97/7.15	-1.30	0.016	150/45/9

1080	clone GSA0864 5'	gi|209929778	*Citrus unshiu*	clementine0.9_020733m	AT3G54420.1	28.52/40.00	6.64/5.90	-1.83	0.030	114/28/6
1081	clone C04010B03	gi|63060920	*Citrus clementina*	clementine0.9_020733m	AT3G54420.1	22.68/40.00	9.55/6.10	-1.81	0.027	110/36/6
1094	PT11-C9-005-004-C03-CT.F	gi|188333275	*Poncirus trifoliata*	clementine0.9_020475m	AT3G12490.2	35.07/39.00	6.69/7.85	1.27	0.048	90/39/9
1102	clone F80DAB0001_ IVF_D04 5'	gi|45449729	*Citrus sinensis*	clementine0.9_021222m	AT2G47730.1	24.55/39.00	6.38/8.30	-1.16	0.025	134/42/9
1104	clone C34003B06	gi|218845933	*Citrus clementina*	clementine0.9_021364m	AT1G78380.1	28.36/40.00	7.07/9.15	1.47	0.005	103/28/11
1108	clone UCRPT02-45F01-L2-1-5.g	gi|57874981	*Citrus trifoliata*	clementine0.9_020929m	AT2G15220.1	27.65/40.00	6.65/8.75	-1.87	0.025	155/41/13
1150	clone CS_REb0004P08	gi|46209000	*Citrus sinensis*	clementine0.9_022586m	AT1G33140.1	30.01/37.00	9.19/10.30	1.17	0.013	229/46/11
1166	clone KN0AAP4YL20	gi|218837597	*Citrus clementina*	clementine0.9_022102m	AT3G22630.1	25.51/35.50	6.34/7.55	-1.42	0.017	282/55/18
1167	clone A1650001_IIF_A05 5'	gi|45451803	*Citrus sinensis*	clementine0.9_017849m	AT3G10920.1	33.39/35.50	8.52/7.80	-1.34	0.023	285/43/14
1188	clone 724 5'	gi|229045058	*Citrus sinensis*	clementine0.9_021335m	AT1G17860.1	28.23/34.50	8.75/5.85	-1.47	0.048	171/44/13
1192	clone 724 5'	gi|229045058	*Citrus sinensis*	clementine0.9_021335m	AT1G17860.1	28.23/34.00	8.75/6.70	-1.79	0.041	293/54/13
1201	5 Y 1069	106422UC4511288		clementine0.9_020989m	AT2G32645.1	39.45/34.00	9.35/5.40	2.12	0.016	125/26/12
1266	clone 724 5'	gi|229045058	*Citrus sinensis*	clementine0.9_021335m	AT1G17860.1	28.23/29.50	8.75/6.70	-1.45	0.026	88/46/10
1280	clone VPE-18_C10 5'	gi|71598314	*Citrus sinensis*	clementine0.9_025138m	AT1G65980.1	19.39/28.00	5.61/6.80	1.20	0.011	109/50/8
1285	clone CS_REb0002M11	gi|46207565	*Citrus sinensis*	clementine0.9_020920m	AT5G10160.1	28.92/29.00	8.78/7.75	1.27	0.011	138/23/6
1325	clone 724 5'	gi|229045058	*Citrus sinensis*	clementine0.9_021335m	AT1G17860.1	28.23/27.00	8.75/8.75	-1.66	0.011	80/32/3
1361	clone KN0AAI3DE08	gi|218825385	*Citrus clementina*	clementine0.9_021495m	AT2G25640.1	26.76/24.00	9.01/6.90	-1.66	0.011	209/40/10
1384	PT11-C1-900-021-E10-CT.F	gi|188320971	*Poncirus trifoliata*	clementine0.9_026418m	AT2G47710.1	34.79/24.00	9.53/8.90	-1.21	0.023	118/22/6
1409	clone USDA-FP_02281 5'	gi|21652359	*Citrus sinensis*	clementine0.9_020696m	AT5G63310.1	19.29/21.00	6.21/7.05	-1.35	0.011	121/43/10
1421	IC0AAA87DD12RM1 CitNFL	gi|110876970	*Citrus clementina*	clementine0.9_020574m	AT4G05180.1	29.05/21.50	9.32/10.25	-1.39	0.049	338/49/15
1428	clone KN0AAP8YO03	gi|218842640	*Citrus clementina*	clementine0.9_019962m	AT4G11600.1	27.03/33.00	8.93/6.95	-1.43	0.021	123/31/8
1429	CR05-C3-700-062-E09-CT.F	gi|188407333	*Citrus reticulata*	clementine0.9_034228m	AT2G45790.1	31.75/42.50	9.20/8.30	1.46	0.016	98/30/8
1432	clone KN0AAM3CH09	gi|218799286	*Citrus sinensis*	clementine0.9_015794m	AT1G24020.2	26.67/31.00	9.34/7.55	-1.57	0.021	110/53/11
1433	putative spindle disassembly related protein CDC48	gi|98962497	*Nicotiana tabacum*	clementine0.9_029783m	AT5G03340.1	90.64/89.00	5.13/6.00	1.22	0.016	170/22/20

a Protein identified by LC-MS/MS.

b Chloroplast precursor.

Table 2.2 List of identified proteins in buds.

Spot	Biological process/**Putative Protein Name**	Accesion no.	Species	*Citrus clementina* Homologue	*Arabidopsis thaliana* Homologue (TAIR gene)	GO Molecular function	Theor./Exp. Mol. Mass (KDa)	Theor./Exp.pI	Ratio	*p*-value	Score/sequence coverage (%)/peptides matched
	(i) Related to primary metabolism Photosynthesis and carbohydrate metabolism										
91	Class III HD-Zip protein HDZ31 [a]	gi|110349530	*Ceratopteris richardii*	Ciclev10007435m.g	AT1G52150.1	DNA binding	86.4/92.0	6.22/8.50	1.46	0.044	41/1/1
164	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic [a]	gi|120669	*Magnolia liliflora*	Ciclev10032014m.g	AT1G13440.1	Nucleotide binding	37.1/82.0	7.10/8.70	1.55	0.025	50/4/2
192	CS00-C3-701-060-H09-CT.F	gi|188269889	*Citrus sinensis*	Ciclev10018991m.g	AT2G45290.1	Transketolase activity	32.7/83.0	5.78/6.50	1.37	0.045	107/38/11
211	Phosphoglucomutase, cytoplasmic [a]	gi|12585330	*Populus tremula*	Ciclev10014721m.g	AT1G23190.1	Intramolecular transferase activity	63.4/80.0	5.49/5.80	1.58	0.034	336/18.2/11
223	Pyrophosphate-dependent phosphofructokinase alpha subunit [a]	gi|3790102	*Citrus x paradisi*	Ciclev10000595m.g	AT1G76550.1	Kinase activity	68.1/78.5	6.71/7.70	1.87	0.019	679/33.3/21
225	Pyrophosphate-dependent phosphofructokinase alpha subunit [a]	gi|3790102	*Citrus x paradisi*	Ciclev10000595m.g	AT1G76550.1	Kinase activity	68.1/78.0	6.71/7.25	1.35	0.041	533/32.8/20
248	NADP-dependent malic enzyme [a]	96943UC451809	Magnoliophyta	Ciclev10025125m.g	AT5G25880.1	Acyltransferase activity	75.9/78.5	8.26/6.25	-1.36	0.039	113/19/12
268	224 LT33-C1-003-105-H04-CT.F	gi|188255904	*Citrus latifolia*	Ciclev10011402m.g	AT1G09780.1	Phosphoglycerate mutase activity	33.7/76.0	9.07/6.30	1.76	0.010	224/38/12
271	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	136018UC385107	*Mesembryanthemum crystallinum*	Ciclev10011402m.g	AT3G08590.2	Phosphoglycerate mutase activity	72.8/76.0	6.68/6.20	1.32	0.016	112/16/12
294	Pyrophosphate-dependent phosphofructokinase beta subunit	gi|162955861	*Citrus sinensis x Poncirus trifoliata*	Ciclev10014760m.g	AT1G12000.1	Kinase activity	62.0/70.5	6.30/7.30	1.55	0.042	176/30/15
297	Glucose-6-phosphate isomerase, cytosolic 2 [a]	gi|1730174	*Clarkia xantiana*	Ciclev10000722m.g	AT5G42740.1	Isomerase activity	63.0/70.0	6.40/7.50	1.41	0.037	148/9.8/6
315	Curculin-like (Mannose-binding) lectin	99164UC454030	*Medicago truncatula*	Ciclev10020212m.g	AT1G78850.1	Carbohydrate binding	55.2/69.0	7.54/6.50	-2.03	0.012	244/27/14
321	Pyruvate kinase [a]	gi|68138979	*Citrus sinensis*	Ciclev10019997m.g	AT5G08570.1	Kinase activity	56.2/68.0	8.34/8.90	1.50	0.026	616/37/20
323	Embryo sac development arrest 9 [a]	gi|15235282	*Arabidopsis thaliana*	Ciclev10025148m.g	AT4G34200.1	Dehydrogenase activity	63.6/69.0	6.16/6.15	1.25	0.030	40/3.8/2
324	Curculin-like (Mannose-binding) lectin	2433UC37CL186Contig5	*Medicago truncatula*	Ciclev10020212m.g	AT1G16905.1	Carbohydrate binding	30.3/69.0	6.24/6.25	-1.52	0.017	138/33/14
339	Pyruvate kinase, cytosolic isozyme [a]	gi|2497543	*Nicotiana tabacum*	Ciclev10019997m.g	AT5G56350.1	Kinase activity	55.8/67.0	6.40/9.35	1.70	0.027	173/9.3/6
353	ATP synthase subunit alpha, mitochondrial [a]	gi|114404	*Helianthus annuus*	Ciclev10012766m.g	ATMG01190.1	ATP synthase activity	55.8/65.0	6.02/6.35	1.14	0.048	516/25.5/15
372	CS00-C3-704-064-E03-	gi|188281687	*Citrus sinensis*	Ciclev10015332m.g	AT5G08690.1	ATPase activity	30.7/64.0	10.69/5.50	-1.27	0.030	106/39/10

373	CT.F UCRCS08_0004I24_r	gi|55396782	*Citrus sinensis*	Ciclev10011726m.g	AT2G36530.1	Enolase activity	30.9/64.0	5.79/6.20	1.49	0.017	182/34/9
398	6-phosphogluconate dehydrogenase [a]	gi|2529229	*Glycine max*	Ciclev10031373m.g	AT3G02360.1	Dehydrogenase activity	56.9/59.0	5.55/7.55	1.79	0.012	477/26.9/14
406	6-phosphogluconate dehydrogenase [a]	gi|2529229	*Glycine max*	Ciclev10031373m.g	AT3G02360.1	Dehydrogenase activity	56.8/59.5	5.55/7.25	1.47	0.011	527/21.5/17
427	6-phosphogluconate dehydrogenase [a]	gi|2529229	*Glycine max*	Ciclev10031373m.g	AT3G02360.1	Dehydrogenase activity	56.9/58.0	5.55/7.25	1.63	0.021	313/14/9
428	Clone ANT0194 5'	gi|209927041	*Citrus unshiu*	Ciclev10011597m.g	AT3G02360.1	Dehydrogenaseactivity	26.2/57.5	9.39/7.75	1.74	0.095	97/29/8
451	NADP-dependent isocitrate dehydrogenase [a]	gi|15982950	*Prunus persica*	Ciclev10014816m.g	AT1G65930.1	Dehydrogenaseactivity	46.9/57.0	6.54/7.90	1.54	0.012	137/10.6/5
505	3-phosphoglycerate kinase [a]	gi|29124969	*Populus tremuloides*	Ciclev10005101m.g	AT1G79550.2	Kinase activity	42.6/54.5	8.78/6.10	1.28	0.028	118/17/6
519	Glyceraldehyde-3-phosphate dehydrogenase B, chloroplastic [a]	gi|120665	*Nicotiana tabacum*	Ciclev10015476m.g	AT1G42970.1	Nucleotide binding	48.0/54.0	8.83/6.80	1.29	0.032	387/24/15
523	Clone UCRCS08-24F08-L16	gi|56585873	*Citrus sinensis*	Ciclev10005101m.g	AT1G79550.2	Kinase activity	28.3/54.0	9.19/6.60	1.58	0.028	292/57/16
530	Pyruvate dehydrogenase	gi|68138987	*Citrus x paradisi*	Ciclev10031744m.g	AT1G59900.1	Dehydrogenaseactivity	44.2/53.0	8.09/7.30	1.20	0.038	70/22/12
532	Cytosolic phosphoglycerate kinase [a]	gi|121485004	*Helianthus annuus*	Ciclev10005101m.g	AT1G79550.2	Kinase activity	42.3/53.5	5.82/6.00	1.19	0.025	701/31/13
542	Cinnamyl alcohol dehydrogenase [a]	gi|122894098	*Citrus sinensis*	Ciclev10028651m.g	AT3G19450.1	Dehydrogenase activity	39.4/53.0	5.88/6.75	1.21	0.023	193/25.5/8
571	Fructose-bisphosphate aldolase	95341UC45207	*Codonopsis lanceolata*	Ciclev10012049m.g	AT2G36460.1	Aldolase activity	58.0/51.0	9.22/8.50	1.45	0.010	406/43/26
578	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic [a]	gi|120661	*Nicotiana tabacum*	Ciclev10015476m.g	AT3G26650.1	Nucleotide binding	42.1/50.05	6.60/7.20	-1.47	0.029	93/10.5/4
582	Clone IC0AAA70BB11	gi|218837076	*Citrus clementina*	Ciclev10032014m.g	AT3G04120.1	Nucleotide binding	35.0/50.5	7.79/8.70	1.51	0.011	287/38/14
590	Aldose 1-epimerase-like protein	14833UC37CL7571Contig1	*Arabidopsis thaliana*	Ciclev10008981m.g	AT3G17940.1	Epimerase activity	42.0/50.5	6.87/6.75	1.09	0.026	94/18/9
601	IC0AAA13BA03RM1 CitNFL	gi|110843365	*Citrus clementina*	Ciclev10017877m.g	AT2G36460.1	Aldolase activity	45.4/49.0	10.20/7.80	1.16	0.043	88/32/14
608	Os06g0666600 [a]	gi|115469420	*Oryza sativa* Japonica	Ciclev10032014m.g	AT1G16300.1	Nucleotide binding	43.7/49.0	8.78/8.10	2.10	0.015	181/15.9/9
623	Volcani_1_04_G01	gi|62426980	*C. reticulata x C. temple*	Ciclev10028831m.g	AT3G12500.1	Chitinase activity	33.7/48.0	4.72/4.20	-2.70	0.031	333/34/11
629	Volcani_1_04_G01	gi|62426980	*C. reticulata x C. temple*	Ciclev10028831m.g	AT3G12500.1	Chitinase activity	33.7/48.0	4.72/4.70	-1.67	0.025	341/29/10
632	Acidic class I chitinase	11343UC37CL4717Contig2	*Citrus jambhiri*	Ciclev10028964m.g	AT3G12500.1	Chitinase activity	44.0/48.0	6.58/4.45	-1.83	0.017	267/30/13
641	Chitinase [a]	gi|1220144	*Citrus sinensis*	Ciclev10028964m.g	AT3G12500.1	Chitinase activity	32.5/48.5	5.06/6.00	-1.28	0.028	49/5.2/2
679	Clone KN0AAP8YP14	gi|218801456	*Citrus clementina*	Ciclev10028831m.g	AT3G12500.1	Chitinase activity	26.6/47.5	4.61/4.45	-1.67	0.012	240/28/7
700	Clone VPE-04_H09 5'	gi|71598939	*Citrus sinensis*	Ciclev10028831m.g	AT3G12500.1	Chitinase activity	18.0/46.5	8.98/4.25	-1.93	0.012	117/44/9
726	Volcani_1_04_G01	gi|62426980	*Citrus reticulata x C. temple*	Ciclev10028831m.g	AT3G12500.1	Chitinase activity	33.7/46.5	4.72/4.30	-1.90	0.012	169/34/11
727	Volcani_1_04_G01	gi|62426980	*Citrus reticulata x Citrus temple*	Ciclev10028831m.g	AT3G12500.1	Chitinase activity	33.7/46.5	4.72/4.50	-1.54	0.034	144/35/9
757	Volcani_1_04_H02	gi|62426991	*Citrus reticulata x Citrus temple*	Ciclev10028831m.g	AT3G12500.1	Chitinase activity	29.5/44.5	4.70/4.95	-1.88	0.015	262/42/7
765	Volcani_1_04_G01	gi|62426980	*Citrus reticulata x Citrus temple*	Ciclev10028831m.g	AT3G12500.1	Chitinase activity	33.7/44.5	4.72/5.30	-1.40	0.029	102/26/7

776	Volcani_1_04_H02	gi|62426991	*Citrus reticulata x Citrus temple*	Ciclev10028831m.g	AT3G12500.1	Chitinase activity	29.5/44.5	4.70/5.20	-2.16	0.017	314/46/9
788	Clone KN0AAA1DE07	gi|218806186	*Citrus clementina*	Ciclev10028964m.g	AT3G12500.1	Chitinase activity	32.9/43.5	5.33/4.35	-2.00	0.030	187/29/7
789	Volcani_2_02_H04	gi|62430478	*Citrus reticulata x Citrus temple*	Ciclev10028959m.g	AT3G12500.1	Chitinase activity	31.5/43.5	5.28/4.70	-2.21	0.012	399/31/8
792	Clone KN0AAA1DE07	gi|218806186	*Citrus clementina*	Ciclev10028964m.g	AT3G12500.1	Chitinase activity	32.9/43.5	5.33/4.35	-1.71	0.025	297/29/7
794	Clone STG0267 5'	gi|209937616	*Citrus unshiu*	Ciclev10028964m.g	AT3G12500.1	Chitinase activity	39.7/43.5	5.81/4.95	-1.70	0.026	381/33/12
801	Clone MWYG276 5'	gi|209935316	*Citrus unshiu*	Ciclev10028964m.g	AT3G12500.1	Chitinase activity	36.8/43.5	5.74/5.25	-1.72	0.015	131/17/6
806	Volcani_2_02_H04	gi|62430478	*Citrus reticulata x Citrus temple*	Ciclev10028959m.g	AT3G12500.1	Chitinase activity	31.5/43.5	5.28/5.10	-1.87	0.019	180/31/8
809	UCRCS07_15A01_g	gi|56528325	*Citrus sinensis*	Ciclev10028831m.g	AT3G12500.1	Chitinase activity	31.3/43.5	5.55/5.65	-1.86	0.012	73/23/5
811	Clone VPE-33_E04 5'	gi|71597495	*Citrus sinensis*	Ciclev10028959m.g	AT3G12500.1	Chitinase activity	25.7/43.5	5.29/5.00	-1.85	0.025	229/41/6
861	Clone IC0AAA42BH10	gi|218810683	*Citrus clementina*	Ciclev10025528m.g	AT2G18193.1	ATPase activity	35.1/41.5	9.87/4.65	-1.63	0.010	106/33/20
875	Clone IC0AAA82CA03	gi|218847452	*Citrus clementina*	Ciclev10005522m.g	AT5G24090.1	Hydrolase activity	35.5/40.0	9.40/9.55	-1.33	0.012	228/32/11
876	Clone IC0AAA49DB04	gi|218792490	*Citrus clementina*	Ciclev10028924m.g	AT2G21170.1	Triose-phosphate isomerase activity	32.9/41.5	8.13/5.15	-1.68	0.012	219/30/9
879	CS00-C3-701-006-D07-CT.F	gi|188239503	*Citrus sinensis*	Ciclev10016206m.g	AT2G32520.1	Hydrolase activity	30.9/41.5	5.80/5.70	-1.45	0.027	111/21/8
881	Clone FBI0356 5'	gi|209923997	*Citrus unshiu*	Ciclev10005522m.g	AT5G24090.1	Hydrolase activity	33.0/40.0	9.18/9.40	-1.21	0.025	131/22/8
926	Clone FLAVEDO0002_IVaR_F01 3'	gi|28716335	*Citrus sinensis*	Ciclev10003750m.g	AT3G54420.1	Chitinase activity	18.1/35.0	9.00/7.80	-1.35	0.012	114/41/11
946	Os05g0295800 [a]	gi|115463027	*Oryza sativa* Japonica	Ciclev10029169m.g	AT1G08110.4	lactoylglutathione lyase activity	21.4/36.0	5.84/5.35	-1.30	0.011	104/12.2/3
1006	Oxygen-evolving enhancer protein 2, chloroplastic a	gi|131390	*Pisum sativum*	Ciclev10032502m.g	AT1G06680.1	Calcium ion binding	28.2/25.0	8.29/9.20	-2.47	0.014	55/5/2
1088	60S ribosomal protein L12 [a]	gi|40287508	*Capsicum annuum*	Ciclev10012802m.g	AT2G37190.1	Nucleic acid binding	17.8/9.0	8.81/9.70	1.63	0.010	182/28/4
1116	CVSOVPOAN969	gi|306800471	*Citrus sinensis*	Ciclev10028959m.g	AT3G12500.1	Chitinase activity	13.0/8.0	9.74/4.60	-2.14	0.017	83/50/7
1157	CR05-C3-700-050-F06-CT.F	gi|188325653	*Citrus reticulata*	Ciclev10016525m.g	AT4G05180.1	Calcium ion binding	30.0/5.0	9.80/9.45	-1.61	0.012	242/50/13
1160	CG32-C1-003-057-C09-CT.F	gi|188395142	*Citrus aurantifolia*	Ciclev10016525m.g	AT4G05180.1	Calcium ion binding	31.0/5.0	10.21/9.25	-1.95	0.012	159/54/13
1163	Nucleoside diphosphate kinase 2, chloroplastic [a]	gi|266607	*Spinacia oleracea*	Ciclev10021889m.g	AT5G63310.1	Kinase activity	26.0/5.0	9.11/6.60	1.25	0.042	58/7/2
1166	Nucleoside diphosphate kinase 2, chloroplastic [a]	gi|266607	*Spinacia oleracea*	Ciclev10021889m.g	AT5G63310.1	Kinase activity	26.0/5.0	9.11/5.90	-1.53	0.011	76/7/2
1227	Fructose-bisphosphate aldolase cytoplasmic isozyme [a]	gi|78099751	*Oryza sativa* Japonica	Ciclev10012049m.g	AT2G36460.1	Aldolase activity	39.2/52.0	6.96/9.05	1.57	0.012	213/17.6/2
	Krebs cycle										
70	Aconitate hydratase, cytoplasmic [a]	gi|1351856	*Cucurbita maxima*	Ciclev10007338m.g	AT2G05710.1	Hydrolyase activity	98.6/92.0	5.74/7.30	1.87	0.047	239/8/8
71	Cytosolic aconitase [a]	gi|11066033	*Nicotiana tabacum*	Ciclev10007338m.g	AT2G05710.1	Hydrolyase activity	98.7/92.0	5.88/7.35	1.91	0.027	210/10.1/11
81	Volcani_8_02_C8 Volcani08	gi|157648401	*Citrus reticulata x Citrus temple*	Ciclev10014140m.g	AT2G05710.1	Hydrolyase activity	31.1/92.0	8.71/7.10	1.69	0.048	158/45/11
82	Clone IC0AAA65CF05	gi|218842914	*Citrus clementina*	Ciclev10014140m.g	AT2G05710.1	Hydrolyase activity	32.5/92.0	9.58/7.15	1.85	0.045	102/21/7
421	Fumarate hydratase 1, mitochondrial [a]	gi|108708038	*Oryza sativa* Japonica	Ciclev10019838m.g	AT5G50950.2	Lyase activity	54.0/58.0	6.93/7.90	1.19	0.041	126/9.4/6
433	Fumarate hydratase 1, mitochondrial [a]	gi|108708038	*Oryza sativa* Japonica	Ciclev10019838m.g	AT5G50950.2	Lyase activity	54.0/57.5	6.93/8.05	1.39	0.021	129/9.2/7
637	Succinyl-CoA synthetase	gi|75294330	*Oryza sativa*	Ciclev10032075m.g	AT5G23250.1	Catalytic activity	34.6/48.5	8.46/8.75	1.31	0.026	264/24.8/11

	subunit alpha; mitochondrial a		*Japonica*								
	Respiration										
933	Putative cytochrome c oxidase subunit II PS17 [a]	gi|109892850	*Pinus strobus*	clementine0.9_018112m[b]	AT4G20400.2	DNA binding	1.7/37.0	9.63/6.00	-1.74	0.012	64/100/2
967	Putative cytochrome c oxidase subunit II PS17 [a]	gi|109892850	*Pinus strobus*	clementine0.9_018112m[b]	AT4G20400.2	DNA binding	1.7/30.5	9.63/7.00	-1.34	0.012	68/100/4
973	Putative cytochrome c oxidase subunit II PS18 [a]	gi|109892851	*Pinus strobus*	clementine0.9_018112m[b]	AT4G20400.2	DNA binding	1.7/28.5	9.63/4.35	-1.37	0.034	63/100/3
991	Putative cytochrome c oxidase subunit II PS17 [a]	gi|109892850	*Pinus strobus*	clementine0.9_018112m[b]	AT4G20400.2	DNA binding	1.7/28.5	9.63/6.20	-1.91	0.012	52/100/2
998	Putative cytochrome c oxidase subunit II PS17 a	gi|109892850	*Pinus strobus*	clementine0.9_018112mb	AT4G20400.2	DNA binding	1.7/25.0	9.63/7.70	-2.24	0.012	57/100/2
1015	Putative cytochrome c oxidase subunit II PS17 a	gi|109892850	*Pinus strobus*	clementine0.9_018112mb	AT4G20400.2	DNA binding	1.7/25.0	9.63/6.20	-1.20	0.047	63/100/4
1034	Putative cytochrome c oxidase subunit II PS17 [a]	gi|109892850	*Pinus strobus*	clementine0.9_018112m[b]	AT4G20400.2	DNA binding	1.7/19.0	9.63/4.25	-1.35	0.012	54/100/4
1042	Putative cytochrome c oxidase subunit II PS17 [a]	gi|109892850	*Pinus strobus*	clementine0.9_018112m[b]	AT4G20400.2	DNA binding	1.7/20.0	9.63/5.75	-2.19	0.010	58/100/2
1054	Putative cytochrome c oxidase subunit II PS17 [a]	gi|109892850	*Pinus strobus*	clementine0.9_018112m[b]	AT4G20400.2	DNA binding	1.7/15.0	9.63/4.35	-1.47	0.026	57/100/2
1074	Putative cytochrome c oxidase subunit II PS17 [a]	gi|109892850	*Pinus strobus*	clementine0.9_018112m[b]	AT4G20400.2	DNA binding	1.7/10.5	9.63/8.90	-1.93	0.012	68/100/2
1090	Putative cytochrome c oxidase subunit II PS17 [a]	gi|109892850	*Pinus strobus*	clementine0.9_018112m[b]	AT4G20400.2	DNA binding	1.7/11.0	9.63/5.70	-1.41	0.011	54/100/2
1152	Putative cytochrome c oxidase subunit II PS17 [a]	gi|109892850	*Pinus strobus*	clementine0.9_018112m[b]	AT4G20400.2	DNA binding	1.7/4.9	9.63/4.25	-1.31	0.042	62/100/2
1172	Putative cytochrome c oxidase subunit II PS17 [a]	gi|109892850	*Pinus strobus*	clementine0.9_018112m[b]	AT4G20400.2	DNA binding	1.7/4.8	9.63/7.20	-1.27	0.026	43/50/3
1214	Putative cytochrome c oxidase subunit II PS17 [a]	gi|109892850	*Pinus strobus*	clementine0.9_018112m[b]	AT4G20400.2	DNA binding	26.0/4.0	9.63/6.25	-1.40	0.039	43/50/1
	(ii) Oxidoreductase activity										
251	Multicopper oxidase, type 1 [a]	gi|124360517	*Medicago truncatula*	Ciclev10015627m.g	AT1G76160.1	Oxidase activity	60.1/76.0	7.71/8.30	1.59	0.033	184/9.1/5
355	Dihydrolipoamide dehydrogenase [a]	13339UC37CL6262Contig1	*Bruguiera gymnorhiza*	Ciclev10004793m.g	AT1G48030.2	Dihydrolipoyl Dehydrogenase activity	64.8/65.0	8.25/7.33	1.35	0.037	200/39/21
455	Clone C31201G12	gi|218785734	*Citrus aurantium*	Orange1.1g0118796m.g[c]	AT5G06720.1	Peroxidase activity	25.9/56.0	7.79/7.75	1.35	0.035	220/31/7
489	CA26-C1-002-067-F11-CT.F	gi|188284956	*Citrus aurantium*	Orange1.1g0118796m.gc	AT5G06720.1	Peroxidase activity	32.2/55.0	9.53/8.40	-1.35	0.012	133/22/5
551	Monodehydroascorbate reductase [a]	gi|15865451	*Mesembryanthemum crystallinum*	Ciclev10011764m.g	AT3G52880.1	Reductase activity	51.9/52.5	6.38/6.65	1.28	0.017	291/8.5/3
594	Clone C06019A01	gi|63067959	*C. clementina x C. tangerina*	Ciclev10028766m.g	AT1G60710.1	Aldo/keto reductase activity	25.0/49.5	6.22/7.30	1.50	0.011	160/47/10
653	Ferredoxin--NADP reductase, chloroplastic [a]	gi|3913650	*Nicotiana tabacum*	Ciclev10015627m.g	AT1G30510.1	Reductase activity	42.3/47.5	8.67/9.30	-1.21	0.028	162/11.2/5
748	clone IC0AAA90AF11	gi|218835758	*Citrus clementina*	Ciclev10001194m.g	AT4G08390.3	Peroxidase activity	33.4/45.5	9.38/6.10	1.28	0.049	128/31/11
749	Chloroplast thylakoid-bound ascorbate peroxidase [a]	gi|45268437	*Vigna unguiculata*	Ciclev10001194m.g	AT1G77490.1	Peroxidase activity	45.2/45.0	8.20/5.75	1.19	0.027	227/13.6/6
828	Clone STG1278 5'	gi|209933573	*Citrus unshiu*	Ciclev10008900m.g	AT5G20080.1	NADH-cytochrome b5 reductase activity	24.2/48.5	9.26/7.80	-1.34	0.016	83/37/9

841	δ-1-pyrroline-5-carboxylate reductase [a]	gi|124359971	*Medicago truncatula*	Ciclev10005607m.g	AT5G14800.1	Reductase activity	31.7/42.0	7.79/9.45	1.26	0.026	73/3/1
870	Clone IC0AAA44CD06	gi|218840820	*Citrus clementina*	Ciclev10030198m.g	AT1G07890.8	Peroxidase activity	31.7/42.0	5.70/5.80	-1.28	0.017	339/55/16
942	Volcani_7_01_F3 Volcani07	gi|157646018	*Citrus reticulata x Citrus temple*	Ciclev10032931m.g	AT1G75270.1	Glutathione S-transferase activity	29.8/36.0	7.84/6.25	-1.28	0.026	291/59/15
974	IC0AAA97DD02RM1 CitNFL	gi|110883047	*Citrus clementina*	Ciclev10012459m.g	AT5G06290.1	Peroxiredoxin activity	37.9/29.0	8.62/4.50	-1.35	0.012	88/22/7
987	SOD [a]	gi|77417707	*Citrus maxima*	Ciclev10032324m.g	AT3G10920.1	Superoxide dismutase activity	15.6/29.0	6.03/6.40	-1.63	0.012	182/22.5/6
989	CR05-C3-702-044-C11-CT.F	gi|188441249	*Citrus reticulata*	Ciclev10032324m.g	AT3G10920.1	Superoxide dismutase activity	30.3/29.0	8.33/6.70	-1.34	0.017	338/42/12
1094	Copper/zinc superoxide dismutase [a]	gi|33340236	*Citrus limon*	Ciclev10029488m.g	AT1G08830.2	Superoxide dismutase activity	1.5/10.0	5.46/6.05	-1.33	0.029	129/24/3
	(iii) Stress/defense responses										
812	Abscisic stress ripening-like protein [a]	gi|38679405	*Glycine max*	Ciclev10002644m.g	AT5G39570.1	N/A	25.3/43.5	5.58/5.75	-1.73	0.039	128/10.1/3
837	Miraculin-like protein [a]	gi|11596178	*Citrus x paradisi*	Ciclev10022041m.g	AT1G17860.1	Endopeptidase inhibitor activity	25.6/42.5	8.11/5.75	-1.36	0.026	149/21.6/5
838	Miraculin-like protein 1 [a]	gi|87299375	*Citrus jambhiri*	Ciclev10022041m.g	AT1G17860.1	Endopeptidase inhibitor activity	25.6/42.5	8.11/6.25	-1.29	0.027	223/31/7
850	Miraculin-like protein 1 [a]	gi|87299375	*Citrus jambhiri*	Ciclev10022041m.g	AT1G17860.1	Endopeptidase inhibitor activity	25.6/42.0	8.11/5.20	-1.49	0.015	424/42.2/11
855	Miraculin-like protein [a]	gi|11596178	*Citrus x paradisi*	Ciclev10022041m.g	AT1G17860.1	Endopeptidase inhibitor activity	25.6/41.5	8.11/4.75	-1.80	0.015	215/30.2/8
890	Miraculin-like protein [a]	gi|11596178	*Citrus x paradisi*	Ciclev10022041m.g	AT1G17860.1	Endopeptidase inhibitor activity	25.6/40.0	8.11/9.20	-1.52	0.045	55/5.2/1
892	Miraculin-like protein [a]	gi|11596178	*Citrus x paradisi*	Ciclev10022041m.g	AT1G17860.1	Endopeptidase inhibitor activity	25.6/41.0	8.11/4.90	-1.49	0.011	147/21.6/5
913	Miraculin-like protein 1 [a]	gi|87299375	*Citrus jambhiri*	Ciclev10022041m.g	AT1G17860.1	Endopeptidase inhibitor activity	25.6/40.0	8.11/5.60	-1.19	0.012	255/31/7
961	Miraculin-like protein 2 [a]	gi|87299377	*Citrus jambhiri*	Ciclev10022041m.g	AT1G17860.1	Endopeptidase inhibitor activity	24.4/30.0	5.61/4.80	-1.84	0.047	204/30.5/7
981	Miraculin-like protein [a]	gi|11596178	*Citrus x paradisi*	Ciclev10022041m.g	AT1G17860.1	Endopeptidase inhibitor activity	25.6/27.5	8.11/7.45	-1.47	0.015	261/29.7/9
1151	Miraculin-like protein [a]	gi|11596178	*Citrus x paradisi*	Ciclev10022041m.g	AT1G17860.1	Endopeptidase inhibitor activity	25.0/6.0	8.11/5.40	-1.86	0.012	195/21/5
1178	Miraculin-like protein 1 [a]	gi|87299375	*Citrus jambhiri*	Ciclev10022041m.g	AT1G17860.1	Endopeptidase inhibitor activity	26.0/4.9	8.11/8.80	-1.72	0.012	153/20/6
	(iv) Signal transduction										
535	Putative ankyrin-repeat protein [a]	gi|37625031	*Vitis aestivalis*	Ciclev10012463m.g	AT4G35450.4	Protein binding	38.1/52.0	4.53/4.20	-2.04	0.011	83/11/4
	(v) Aminoacid metabolism and transport										
130	5-Methyltetrahydropteroyl triglutamate-homocysteine methyltransferase	95371UC45237	*Arabidopsis thaliana*	Ciclev10018965m.g	AT5G17920.2	S-methyl transferase activity	98.0/86.0	7.01/7.50	1.53	0.042	111/16/14
135	Methionine synthase [a]	gi|6716760	*Coffea arabica*	Ciclev10018965m.g	AT5G17920.2	S-methyl transferase activity	24.6/86.0	5.69/7.80	1.57	0.027	44/6.4/1
144	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	95371UC45237	*Arabidopsis thaliana*	Ciclev10018965m.g	AT5G17920.2	S-methyl transferase activity	98.0/86.0	7.01/7.75	2.04	0.024	181/17/15
149	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	568UC37CL1 Contig569	*Arabidopsis thaliana*	Ciclev10018965m.g	AT5G17920.2	S-methyl transferase activity	101.2/86.0	8.19/7.65	1.60	0.031	122/16/14
166	Os03g0242900 [a]	gi|297600633	*Oryza sativa* Japonica	Ciclev10027859m.g	AT4G34980.1	Serine-type peptidase activity	80.7/82.5	7.29/9.30	1.93	0.018	78/3/2
169	Os03g0242900 [a]	gi|297600633	*Oryza sativa* Japonica	Ciclev10027859m.g	AT4G34980.1	Serine-type peptidase activity	80.7/82.5	7.29/9.40	2.54	0.012	72/3.1/2
224	Formate-tetrahydrofolate ligase [a]	gi|18403095	*Arabidopsis thaliana*	Ciclev10014585m.g	AT1G50480.1	Ligase activity	68.3/78.5	6.26/7.35	1.48	0.029	260/8.4/8
288	Putative dihydroxyacid	gi|14532594	*Arabidopsis thaliana*	Ciclev10004565m.g	AT3G23940.1	Hydro-lyase activity	65.5/71.0	5.95/6.80	1.20	0.033	81/6/4

	dehydratase [a]										
301	Leucine-rich repeat transmembrane protein kinase [a]	gi|15226197	*Arabidopsis thaliana*	Ciclev10014433m.g	AT2G01210.1	Protein kinase activity	79.3/70.0	5.75/7.70	1.41	0.012	52/1.1/2
381	Wheat adenosylhomocysteinase-like protein [a]	gi|29367605	*Oryza sativa* Japonica	Ciclev10004852m.g	AT4G13940.1	Hydrolase activity	53.9/63.5	5.62/6.45	1.38	0.026	467/21/14
392	Wheat adenosylhomocysteinase-like protein [a]	gi|29367605	*Oryza sativa* Japonica	Ciclev10004852m.g	AT4G13940.1	Hydrolase activity	53.9/63.0	5.62/6.30	1.29	0.024	344/16.3/10
539	Hypothetical protein [a]	gi|147775474	*Vitis vinifera*	Ciclev10024078m.g	AT1G53280.1	Protein kinase activity	46.0/53.0	9.02/6.50	1.45	0.011	141/7.5/4
552	Aspartate aminotransferase, cytoplasmic	100350UC455216	*Daucus carota*	Ciclev10008489m.g	AT5G11520.1	Transferase activity	63.5/52.0	9.08/9.30	1.27	0.026	162/34/19
565	Serpin-like protein [a]	gi|26224736	*Citrus x paradisi*	Ciclev10001434m.g	AT1G47710.1	Serine-type endopeptidase inhibitor activity	42.4/51.5	6.04/5.95	1.16	0.012	225/15.2/7
566	Serpin-like protein [a]	gi|26224736	*Citrus x paradisi*	Ciclev10001434m.g	AT1G47710.1	Serine-type endopeptidase inhibitor activity	42.4/51.5	6.04/6.20	1.32	0.012	345/15/7
610	Clone LLL1478 5' [a]	gi|209935205	*Citrus limon*	Ciclev10005251m.g	AT5G54160.1	Methyltransferase activity	50.3/49.5	8.99/5.70	1.33	0.026	154/18.4/7
645	Putative chloroplast cysteine synthase 1 [a]	gi|76556492	*Nicotiana tabacum*	Ciclev10020662m.g	AT3G59760.1	Cysteine synthase activity	39.4/48.5	4.84/5.35	-1.21	0.025	68/11.3/4
649	Cysteine synthase [a]	gi|126508778	*Glycine max*	Ciclev10001835m.g	AT4G14880.4	Cysteine synthase activity	34.6/48.5	5.53/5.70	-1.17	0.031	68/8.9/3
673	Tetrahydrofolate dehydrogenase/ cyclohydrolase [a]	gi|15230449	*Arabidopsis thaliana*	Ciclev10021378m.g	AT3G12290.1	Hydrolase activity	31.8/47.5	8.27/8.50	1.53	0.029	104/5.7/3
680	Cysteine synthase [a]	gi|18252506	*Glycine max*	Ciclev10001835m.g	AT4G14880.4	Cysteine synthase activity	34.4/47.5	5.69/5.70	-1.22	0.034	141/15.4/5
692	Gamma-glutamyl hydrolase	99696UC454562	*Arabidopsis thaliana*	Ciclev10020608m.g	AT1G78680.1	Hydrolase activity	52.2/47.0	9.23/7.15	1.24	0.037	203/16/9
697	Clone KN0AAI3DB11	gi|218808173	*Citrus clementina*	Ciclev10021719m.g	AT1G78660.3	Hydrolase activity	29.8/47.0	9.09/6.80	-1.18	0.049	190/33/11
741	Os01g0847900	2659UC37CL242 Contig2	*Oryza sativa* Japonica	Ciclev10012766m.g	AT3G61500.1	Transmembra ne transporter activity	46.5/46.0	11.59/6.30	1.22	0.037	91/41/22
804	CS00-C1-650-045-B12-CT.F[a]	gi|188412227	*Citrus sinensis*	Ciclev10011434m.g	AT3G01120.1	Cystathionine gammasynthase	29.3/43.0	12.01/7.70	-1.36	0.010	49/3/1
831	Cysteine protease [a]	gi|151547430	*Citrus sinensis*	Ciclev10008747m.g	AT5G60360.1	cysteine-type peptidase activity	40.0/42.0	6.25/4.35	-1.65	0.012	179/9.1/3
	(vi) Protein metabolism										
209	CS00-C3-703-102-E05-CT.F	gi|188390232	*Citrus sinensis*	Ciclev10027981m.g	AT5G02500.2	Protein folding (chaperone)	36.3/80.0	9.55/5.00	1.45	0.023	114/25/8
274	Chaperonin putative [a]	gi|15229866	*Arabidopsis thaliana*	Ciclev10014771m.g	AT3G11830.1	Protein folding	60.2/72.5	6.03/7.80	2.04	0.033	363/17.8/10
283	Chaperonin hsp60 [a]	gi|16221	*Arabidopsis thaliana*	Ciclev10014771m.g	AT3G23990.1	Protein folding	61.6/72.5	5.66/5.45	1.27	0.026	727/26/32.6
284	Chaperonin CPN60 mitochondrial [a]	CH60A_ARATH	*Arabidopsis thaliana*	Ciclev10014771m.g	AT3G23990.1	Protein folding	61.6/72.5	5.66/5.10	1.27	0.026	44/5.2/2
352	PT11-C9-005-028-G11-CT.F	gi|188373061	*Citrus trifoliata*	Ciclev10007834m.g	AT4G30920.1	Peptidase activity	29.9/65.0	9.25/6.00	1.22	0.031	134/45/11
472	26S proteasome AAA-ATPase subunit, putative [a]	gi|15241241	*Arabidopsis thaliana*	Ciclev10008422m.g	AT5G20000.1	ATP binding	47.3/55.0	8.69/9.30	1.64	0.026	187/14.3/7
507	Clone KN0AAP7YH01	gi|218791615	*Citrus clementina*	Ciclev10001384m.g	AT1G45000.1	Hydrolase activity	25.0/54.0	6.02/9.00	1.68	0.032	86/47/13
839	CS00-C3-700-007-H09-CT.F	gi|188418361	*Citrus sinensis*	Ciclev10026352m.g	AT2G05840.1	Endopeptidase activity	34.1/42.5	9.09/6.80	1.34	0.029	134/35/11
868	Proteasome subunit alpha type-5 [a]	gi|12229923	*Glycine max*	Ciclev10009299m.g	AT3G14290.1	Peptidase activity	26.1/41.0	4.70/4.25	-1.45	0.026	95/16.9/4
883	Proteasome subunit alpha	gi|12229923	*Glycine max*	Ciclev10009299m.g	AT3G14290.1	Peptidase activity	26.1/40.0	4.70/4.30	-1.46	0.019	399/21.5/9

	type-5 [a]										
908	Chaperonin 10 [a]	gi|3057150	*Arabidopsis thaliana*	Ciclev10009281m.g	AT5G20720.3	Protein folding	26.9/40.0	8.86/5.20	-1.21	0.017	53/6.7/2
930	PT11-C1-900-097-E03-CT.F	gi|188386520	*Citrus trifoliata*	Ciclev10028869m.g	AT1G56450.1	Endopeptidase activity	34.4/34.0	8.90/8.15	1.27	0.012	87/28/7
1061	Predicted protein [CCE9901] [a]	gi|145341867	*Ostreococcus lucimarinus*	Ciclev10029640m.g	AT5G59970.1	DNA-binding (histone)	11.4/10.0	9.25/9.65	1.44	0.026	46/1/1
1063	Flagellar associated protein [a]	gi|159489390	*Chlamydomonas reinhardtii*	Ciclev10030514m.g	AT5G37590.1	Translation initiation factor activity	81.4/13.0	6.41/7.40	1.65	0.010	57/1/1
1078	Initiation factor eIF5-A [a]	gi|13094963	*Manihot esculenta*	Ciclev10032956m.g	AT1G13950.1	Translation initiation factor activity	1.8/13.0	5.6/6.25	1.29	0.031	150/26/4
1079	Peptidyl-prolyl cis-trans isomerase	gi|3334157	*Phaseolus vulgaris*	Ciclev10029386m.g	AT2G21130.1	Peptidyl-prolyl isomerase activity	18.5/10.0	8.36/9.55	1.16	0.050	141/27/5
1112	Volcani_7_04_A4 Volcani07	gi|157646466	*Citrus reticulata x Citrus temple*	Ciclev10029386m.g	AT2G16600.1	Peptidyl-prolyl isomerase activity	32.6/8.0	9.85/9.50	1.68	0.028	157/36/16
1228	RNA polymerase subunit [a]	gi|514322	*Arabidopsis thaliana*	Ciclev10008413m.g	AT1G60850.1	DNA binding	42.2/82.5	5.48/9.00	2.05	0.018	42/2.1/3
	(vii) Mevanolate pathway										
516	Cucumis acetyl-CoA acyltransferase[a]	gi|2494125	*Arabidopsis thaliana*	Ciclev10020068m.g	AT1G04710.1	Acetyl-CoA acyltransferase	49.1/53.5	8.78/9.35	1.53	0.026	76/9/4
517	Putative thiolase	3941UC37CL624 Contig2	*Oryza sativa*	Ciclev10020068m.g	AT2G33150.1	Acetyl-CoA acyltransferase	61.4/53.5	9.28/9.45	1.96	0.095	84/30/14
518	Putative thiolase	3941UC37CL624 Contig2	*Oryza sativa*	Ciclev10020068m.g	AT2G33150.1	Acetyl-CoA acyltransferase	61.4/53.5	9.28/9.55	2.25	0.095	86/30/14
	(viii) Flavonoid biosynthesis										
882	Chalcone--flavonone isomerase [a]	gi|75217175	*Citrus sinensis*	Ciclev10032697m.g	AT3G55120.1	Chalcone isomerase activity	24.0/40.5	5.03/4.75	-1.41	0.023	70/9.5/2
	(ix) Cell wall metabolism										
466	Pectin methylesterase inhibitor PPE8B [a]	gi|6093744	*Prunus persica*	Ciclev10007993m.g	AT4G33220.1	Pectinestesase activity	57.9/55.5	6.16/9.45	2.57	0.010	100/14.2/7
625	Pectinesterase [a]	gi|2578440	*Pisum sativum*	Ciclev10004742m.g	AT1G11580.1	Pectinesterase activity	61.0/48.5	8.77/9.60	1.97	0.043	107/3.6/3
	(x) Other proteins (unknown biological processes)										
463	CS00-C5-003-015-D01-CT.F	gi|188400757	*Citrus sinensis*	Ciclev10000425m.g	AT1G21680.1	N/A	30.5/57.5	11.49/6.50	-1.45	0.018	144/36/8
820	Clone CP45_D09_075 3'	gi|94441763	*Citrus paradisi*	Ciclev10026223m.g	AT4G25150.1	Acid phosphatase activity	18.4/43.0	9.76/9.20	1.26	0.049	92/43/8
863	Clone CS_REa03A10	gi|38035194	*Citrus sinensis*	Ciclev10022041m.g	AT1G17860.1	endopeptidase inhibitor activity	27.6/42.0	8.10/5.55	-1.55	0.012	161/27/5
864	Clone C02002D02	gi|63057463	*Citrus clementina*	Ciclev10022064m.g	AT1G17100.1	N/A	19.8/41.0	5.79/4.35	-1.28	0.010	97/27/5
867	Clone CS_REa03A10	gi|38035194	*Citrus sinensis*	Ciclev10022041m.g	AT1G17860.1	endopeptidase inhibitor activity	27.6/41.5	8.10/8.10	1.57	0.033	558/60/16
873	Clone KN0AAB1BC08	gi|218813857	*Citrus clementina*	Ciclev10022041m.g	AT1G17860.1	endopeptidase inhibitor activity	20.3/40.5	9.78/8.25	1.46	0.042	164/54/11
878	CR05-C3-700-116-F11-CT.F	gi|188243382	*Citrus reticulata*	Ciclev10022001m.g	AT1G17860.1	endopeptidase inhibitor activity	32.4/41.0	10.20/5.30	-1.79	0.016	111/21/8
897	Clone CS_REa03A10	gi|38035194	*Citrus sinensis*	Ciclev10022041m.g	AT1G17860.1	endopeptidase inhibitor activity	27.6/40.0	8.10/7.25	1.63	0.027	509/65/18
898	Clone T24DAB0002_IVF_D12 5'	gi|45448993	*Citrus sinensis*	Ciclev10022001m.g	AT1G17860.1	endopeptidase inhibitor activity	26.4/40.0	8.70/6.90	1.81	0.019	160/28/8
905	Clone CS_REa03A10	gi|38035194	*Citrus sinensis*	Ciclev10022001m.g	AT1G17860.1	endopeptidase inhibitor activity	27.6/39.0	8.10/8.10	1.94	0.016	244/60/14
993	CS00-C5-003-015-D01-CT.F	gi|188400757	*Citrus sinensis*	Ciclev10000425m.g	AT1G21680.1	N/A	30.5/26.0	11.49/7.60	-2.56	0.011	115/20/5
995	CR05-C3-700-055-F04-CT.F [a]	gi|188306468	*Citrus reticulata*	Ciclev10000425m.g	AT1G21680.1	N/A	29.3/26.0	7.88/7.20	-1.73	0.011	97/16.6/3
1007	Clone VPE-33_H06 5' [a]	gi|71597549	*Citrus sinensis*	Ciclev10022211m.g	AT1G17860.1	endopeptidase inhibitor activity	24.7/25.0	8.48/8.55	-5.18	0.013	88/37/7
1030	Clone UCRCS09-8F11-	gi|56538149	*Citrus sinensis*	Ciclev10022211m.g	AT1G17860.1	endopeptidase inhibitor activity	28.6/20.5	9.04/8.60	-2.36	0.042	164/43/11

	K21-1-6.g										
1031	Clone VPE-33_H06 5'	gi|71597549	*Citrus sinensis*	Ciclev10022211m.g	AT1G17860.1	endopeptidase inhibitor activity	24.7/20.5	8.48/8.85	-2.69	0.032	309/60/14
1036	Clone VPE-37_F04 5'	gi|71597528	*Citrus sinensis*	Ciclev10022211m.g	AT1G17860.1	endopeptidase inhibitor activity	24.8/20.0	8.85/7.20	-3.81	0.012	264/43/10
1038	Clone VPE-33_H06 5'	gi|71597549	*Citrus sinensis*	Ciclev10022211m.g	AT1G17860.1	endopeptidase inhibitor activity	24.7/20.0	8.48/8.10	-3.06	0.021	502/68/15
1041	Volcani_8_20_E2 Volcani08	gi|157648131	*Citrus sinensis*	Ciclev10022211m.g	AT1G17860.1	endopeptidase inhibitor activity	30.7/21.0	9.18/6.40	-1.99	0.012	254/38/12
1113	Clone CS_REa03A10	gi|38035194	*Citrus sinensis*	Ciclev10022041m.g	AT1G17860.1	endopeptidase inhibitor activity	27.6/8.0	8.10/9.60	2.30	0.017	174/31/9
1153	Hypothetical protein [a]	gi|3687246	*Arabidopsis thaliana*	Ciclev10010570m.g	AT2G19710.1	N/A	103.0/5.0	5.98/4.90	-2.12	0.017	57/1.6/2
1180	Clone CS_REa05D20	gi|38037650	*Citrus sinensis*	Ciclev10022041m.g	AT1G17860.1	endopeptidase inhibitor activity	28.0/4.8	6.64/9.50	1.86	0.012	107/29/9
1184	Clone CS_REa03A10	gi|38035194	*Citrus sinensis*	Ciclev10022041m.g	AT1G17860.1	endopeptidase inhibitor activity	28.0/4.8	8.10/9.60	2.89	0.095	102/34/11

a Protein identified by LC-MS/MS.

b Chloroplast precursor.

2.3 Classification of identified proteins

The proteins identified in leaves can be classified into seven groups according to their biological function: (i) primary metabolism (33 spots: 26 spots being associated with photosynthesis and carbohydrate metabolism, 4 spots related to Krebs cycle, 1 spot related to pentose phosphate pathway, and 2 spots related to nutrient reservoir activity); (ii) oxidoreductase activity (8 spots: one of them, spot 79, up-regulated in OFF samples, presented the highest ratio among all spots, and 5 spots were catalase, all of them up-regulated in ON-crop samples, with spots 381, 384, 386 and 394 matching the same EST sequence); (iii) stress responses (3 spots, all up-regulated in ON samples); (iv) signal transduction (1 spot); (v) protein synthesis and degradation (6 spots); (vi) expansins (1 spot); (vii) other proteins (58 spots: this is the largest group, most of these proteins with unknown functions). The relative percentages of proteins both in ON leaves and in OFF leaves are given in **Fig. 2.2**.

On the one hand, some of these spots were identified as the same protein such as catalase, for spots 381, 384, 386 and 394 (oxidoreductase group); NADP-isocitrate dehydrogenase, for spots 515 and 516 (Krebs cycle subgroup, up-regulated in ON samples); RuBisCO large subunit-binding protein subunit beta chloroplast, for spots 300, 301 and 307; granule-bound starch synthase Ib precursor, for spots 327 and 328 (**Fig. 2.3**); putative cinnamoyl-CoA reductase, for spots 743, 744 and 748. The last three groups of proteins are related to primary metabolism and all of them are up-regulated in OFF samples. On the other hand, some of the spots were identified as the same protein, but displayed different pI and molecular mass values and might account for isoforms or post-translationally modified forms of these proteins. Examples of the these spots are miraculin-like protein 1 (spots 1016 and 1049) or NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (spots 420 and 437), both related to carbohydrate metabolism, or protein disulphide isomerase (spots 104 and 126, belonging to the protein synthesis and degradation group), all of them up-regulated in OFF samples.

The proteins identified in buds were classified into 10 groups according to biological function involving each protein (**Table 2.2**): (i) primary metabolism (91 spots: 70 spots associated with photosynthesis and carbohydrate metabolism, 7 spots related to Krebs cycle, and 14 spots related to respiration); (ii) oxidoreductase activity (17 spots); (iii) stress/defense responses (12 spots); (iv) signal transduction (1 spot); (v) amino acid metabolism (25 spots); (vi) protein metabolism (18 spots); (vii) the

mevalonate pathway (3 spots); (viii) flavonoid biosynthesis (1 spot); (ix) cell wall metabolism (2 spots); (x) other proteins (22 spots; these proteins are involved in unknown biological processes). The relative percentages of proteins in both the ON and the OFF buds are given in **Fig. 2.2**.

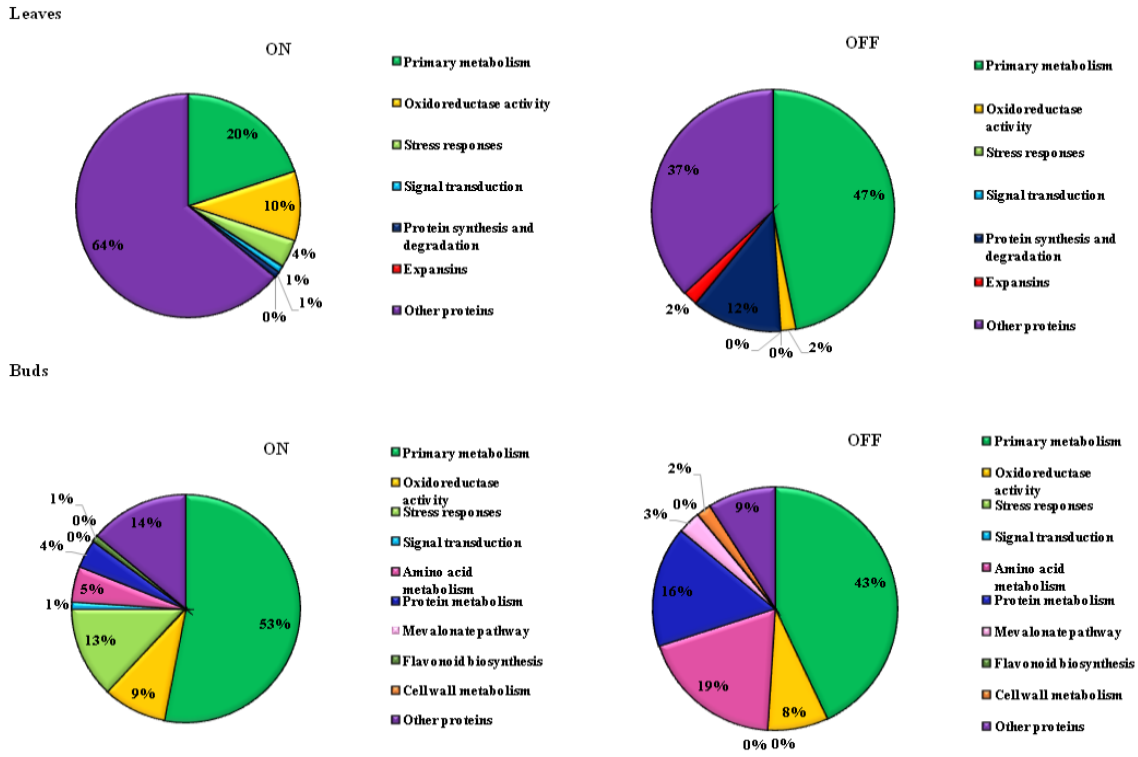


Figure 2.2 Functional classification of the proteins identified and found to be upregulated in ON-crops or OFF-crop leaves and buds. The relative percentages of proteins in each category are shown.

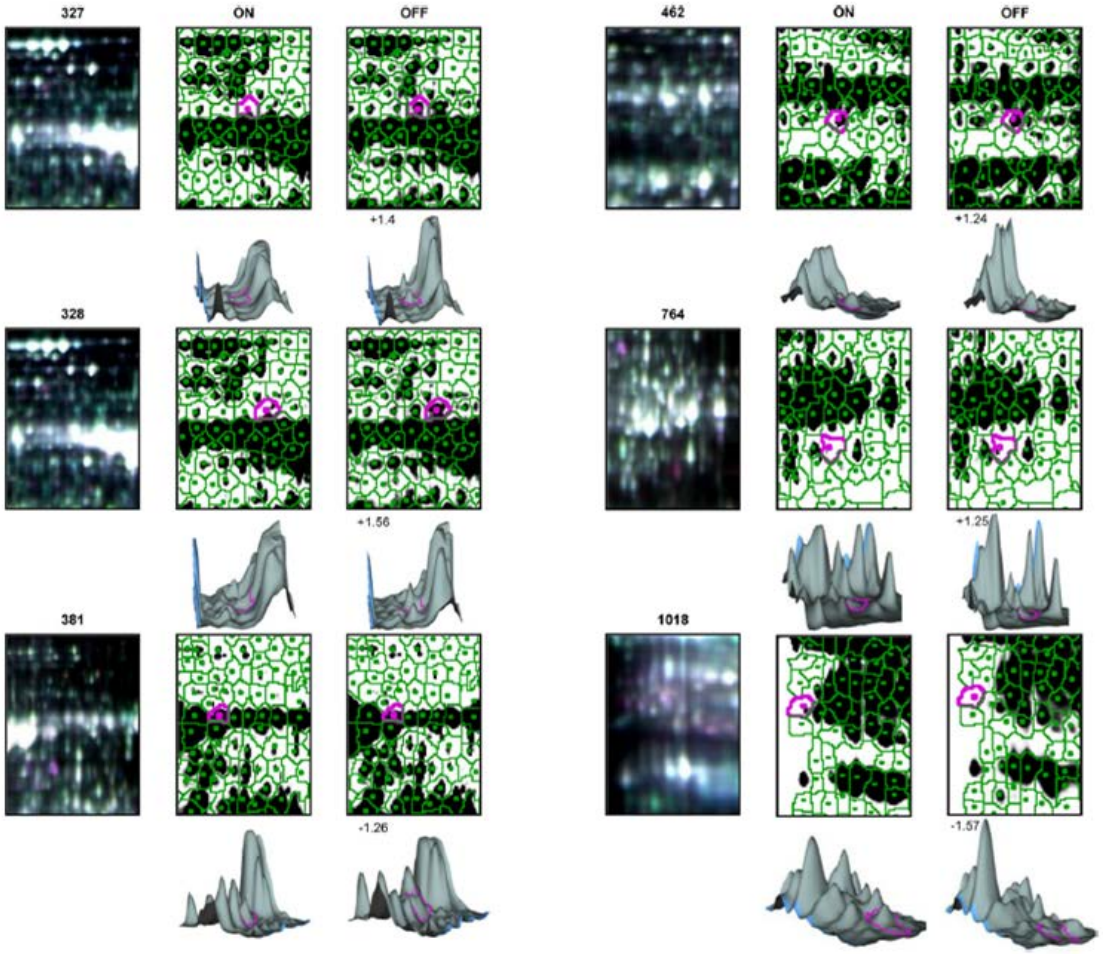


Figure 2.3 Representative proteins analyzed using DeCyder Software (spots 327, 328, 381, 462, 784 and 1018). Differential expression analysis in ON and OFF leaves of representative spots from **Fig. 2.1A and 2.1B.**

2.4 Species from which the identified proteins proceed

Fifty-nine spots were identified in leaves when matched against *Citrus* sequences, the number of matched peptides being between 3 and 24, with 14-70% sequence coverage. Results of a similar order were found for the other 51 proteins, identified by homology to sequences from other species such as *Arabidopsis thaliana* (15 spots), *Prunus persica* (5 spots), *Medicago truncatula* (3 spots), *Vitis vinifera* (2 spots), *Phaseolus vulgaris* (2 spots) or *Solanum lycopersicum* (2 spots).

Ninety-nine spots were identified in buds by matching the *Citrus* sequences, and the number of matched peptides was between 1 and 21, with 5.2-68% sequence coverage (mean 32.44). Results of a similar order were found for the other 93 proteins,

identified by homology with sequences from other species such as *Pinus strobes* (14 spots), *Arabidopsis thaliana* (13 spots), *Oryza sativa* (13 spots) and *Glycine max* (8 spots).

2.5 Gene ontology analysis

Total amounts of protein isolated in leaves were analyzed separately in two groups established according to ratio expression, using the Web tool FatiGO (http://babelomics.bioinfo.cipf.es). The database in www.arabidopsis.orgs was used to search for *Arabidopsis* proteins homologous to proteins identified in this study (**Table 2.1 and Table 2.2**). The establishment of these homologies allowed to know the main biological processes in which the identified proteins are involved. The largest groups of proteins with AV ratio + were composed of proteins involved in carbohydrate and starch biosynthesis, carbohydrate metabolism, protein folding and response to metal ion in the OFF-crop samples. The largest group of proteins with AV ratio - was composed of proteins involved in hydrogen peroxide catabolic process and in response to stress, but many other biological processes are related to the up-expressed proteins in the ON-crop samples (**Fig. 2.4 leaves**).

The ontology study for these proteins in buds revealed that the largest groups of proteins that were up-expressed in the OFF bud samples in November (AV ratio +), as compared to the ON bud samples (AV ratio -), were composed of the proteins not only involved in carbohydrate and amino acid metabolism, but also those expressed in response to stimuli, such as reactive oxygen species (**Fig. 2.4 buds**). The largest group of the proteins with an AV ratio - was made up of the proteins involved in carbohydrate metabolism and also those expressed mainly in response to oxidative stress.

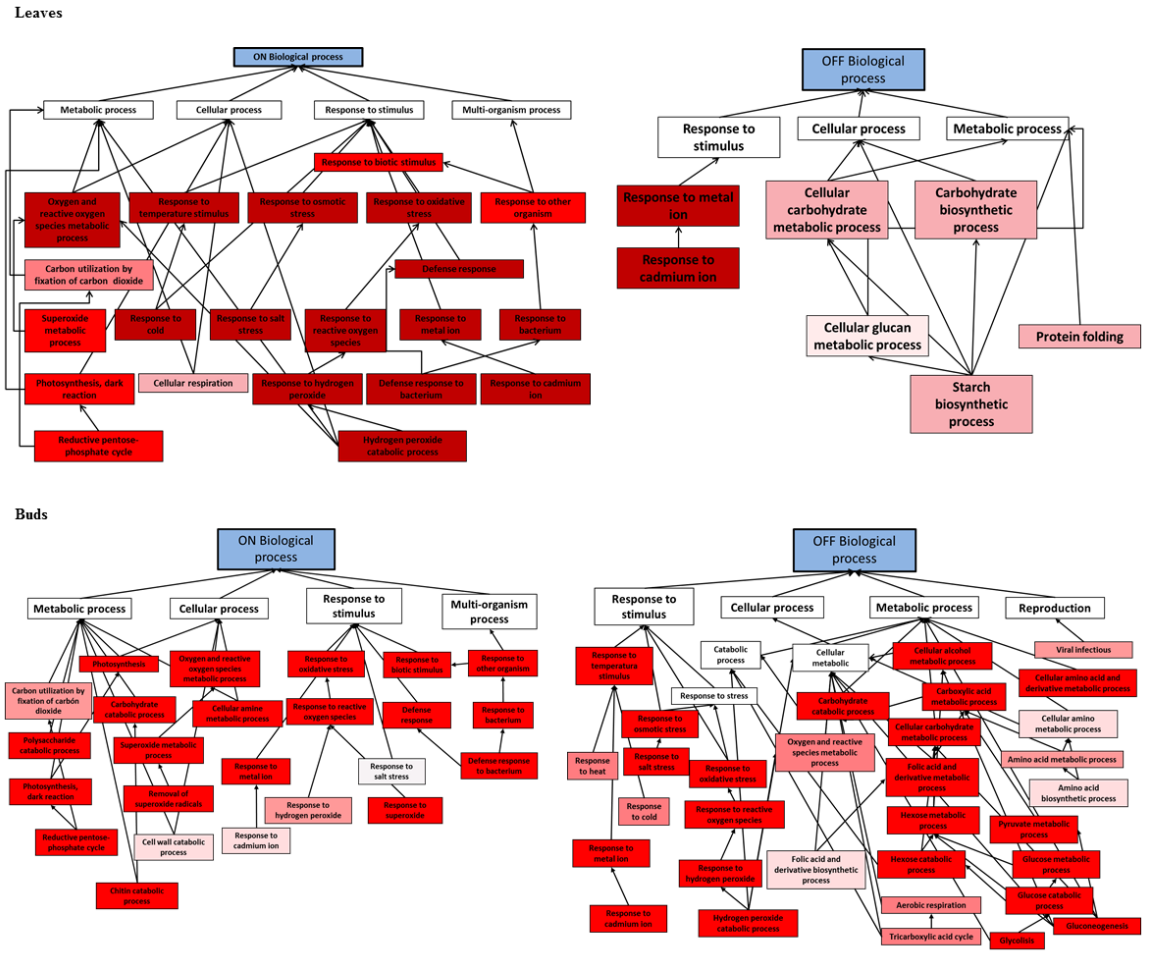


Figure 2.4 Biological processes related to proteins up-regulated in ON and OFF leaves and buds according to FatiGO study. Terms at higher levels of the hierarchy describe more general processes while terms at lower levels are more specific. Color intensity in the graph squares reflects the level of contribution to the total biological processes (the more intense the color, the higher the level of contribution).

2.6 Starch, ABA and JA contents and catalase activity in ON and OFF trees

According to the main biological functions found to be different between ON and OFF trees (primary metabolism, oxidoreductase activity and stress response), starch concentration, catalase activity and ABA and JA concentrations were studied.

The largest group of proteins up-expressed in the OFF-tree leaves compared to the ON-tree leaves, is involved in carbohydrate and starch biosynthesis, both of them related to primary metabolism. Among them, the following proteins may be highlighted: Granule-bound starch synthase Ib precursor (GBSS, spots 327 and 328), malate dehydrogenase glyoxysomal precursor (MD, spot 764) and ADP-glucose pyrophosphorylase small subunit (AGP, spot 462) (**Fig. 2.3**). The activation state of these proteins is correlated with the accumulation of starch and soluble sugar in several tissues from rice, wheat or tomato (Kawasaki *et al.,* 1996; Smidansky *et al.,* 2007; Centeno *et al.,* 2011). In fact, the starch level was found to be significantly higher in OFF leaves than in ON leaves, being these results consistent with the up-expression of GBSS in OFF-tree leaves (**Fig. 2.5A**).

For proteins up-expressed in the ON leaves, the largest groups are those involving a hydrogen peroxide catabolic process, such as catalase (spots 381, 382, 384, 386 and 394) or monodehydroascorbate reductase (spot 474). This is consistent with studies in apple that showed how catalase activity remained high during stages of fruit growth (Abassi *et al.,* 1998). To validate this in *Citrus*, the catalase activity was measured in ON and OFF leaves; the results of this measurement confirmed that catalase activity in ON samples was twice as high that in OFF samples (**Fig. 2.5B**).

Although to a lower extent, the stress responses were also differentially expressed in ON and OFF trees. ABA and JA are related with stress. In fact, an Abscisic acid stress ripening-like protein (spot 802) was found to be up-regulated in ON buds. The quantification of ABA in OFF and ON tissues showed significant differences (**Fig. 2.6**). The highest ABA content in the fruit exocarp was found at the end of November, coinciding with fruit color change, and then the ABA content significantly decreased (**Fig. 1.3, Section 1**). At the same time that the ABA content increased in the exocarp it also increased significantly in buds and leaves (**Fig 2.6**). On the other hand, the ABA content significantly decreased only in OFF leaves and was maintained almost constant in buds (**Fig. 2.6**).

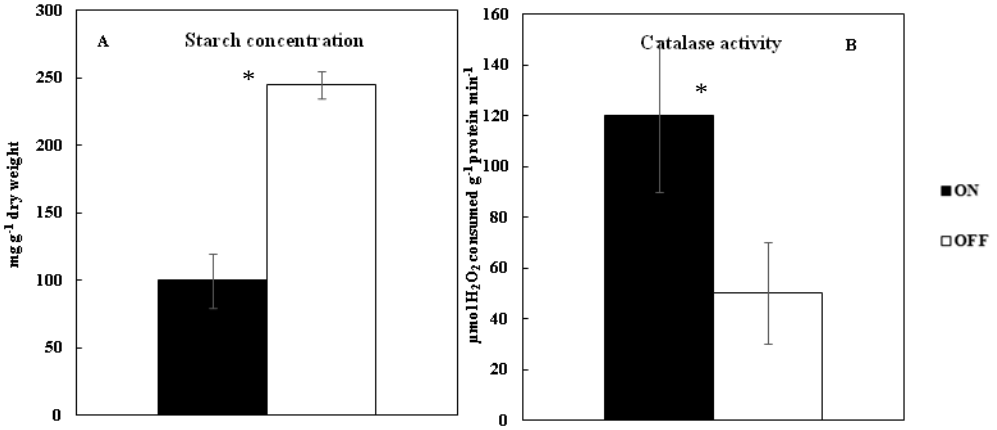


Figure 2.5 A. Starch concentration in ON and OFF leaves expressed in mg g^{-1} dry weight. **B.** Catalase activity in ON and OFF leaves expressed in µmol H_2O_2 consumed g^{-1} protein min^{-1}. Data are means of six independent replicates (n = 6). There are significant differences between ON and OFF leaves ($p \leq 0.05$). *: indicate significant differences.

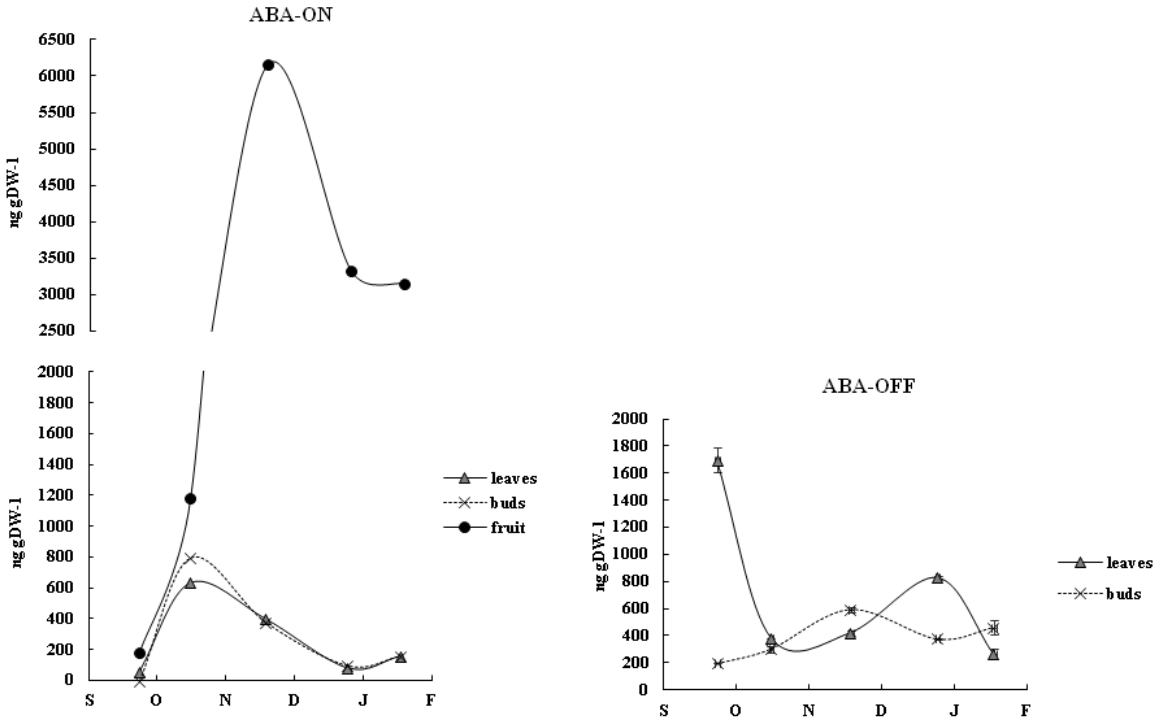


Figure 2.6 Endogenous abscisic acid content in the ON and OFF trees of *C. clementine* cvs. 'Moncada'. ABA was measured in the spring+summer (SP+S) leaves (ON and OFF), fall leaves (OFF), buds (ON and OFF) and exocarp (ON). Data are means ± ES of 2 sets of 10 shoots.

Miraculin-like proteins have been related with defense responses and JA (Maserti *et al.*, 2011), and were up-regulated in ON buds (**Table 2.2**). The JA content showed significant differences between ON and OFF tissues (**Fig. 2.7**). ON shoots showed higher JA content in buds in October and in leaves at the end of November compared to the other tissues at the same time. In the case of the OFF shoots, the JA content showed significant differences between buds and leaves during the entire period of the study. But the highest differences were observed from September to November (2500 ng gDW^{-1} in buds and 1000-250 ng gDW^{-1} in leaves). After November JA content decreased in both tissues. The JA content was higher in OFF than in ON shoots.

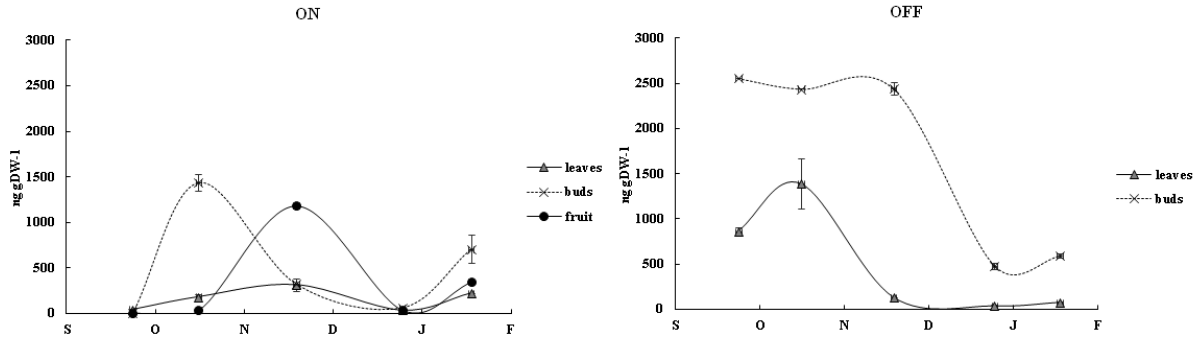


Figure 2.7 Endogenous jasmonic acid content in ON and OFF 'Moncada' trees. JA was measured in the leaves, buds, and fruit exocarp. Data are means ± ES of 2 sets of 10 shoots.

Section 3: Epigenetic control of flowering

3.1 Alternate bearing: biennial reproductive-vegetative development cycle

Citrus alternate bearing trees repeat biennial cycles of reproductive-vegetative development (for more details, see **Figure 4** in the introduction). Alternation of the reproductive development in the 'Moncada' mandarin is shown in **Fig. 3.1**. Twelve trees were selected in summer according to their fruit yield in the year 1: trees 1-6 were ON (red, **Fig. 3.1 A**) whereas trees 7-12 were OFF (blue, **Fig. 3.1 B**). The presence of fruit in the year 1 (red trees) drastically inhibited the number of flowers produced in spring (0.7 flowers/100 nodes) in year 2 compared to OFF trees (blue) (**Fig. 3.1 C and D**). Blue trees flowered profusely (142 flowers/100 nodes) and set a high number of fruits in year 2 (**Fig. 3.1 B**) which inhibited flowering in year 3 (**Fig. 3.1 D**). On the other hand, red trees were OFF in year 2, set a very low yield (**Fig. 3.1 A**), and developed 5300 vegetative shoots $tree^{-1}$ (**Fig. 3.2**), thus producing a high number of new buds receptive to exogenous flowering signals. Accordingly, red trees flowered profusely in year 3 (145 flowers/100 nodes) becoming ON trees again (**Fig. 3.1 A and B**).

The relative expression of the flowering promoters *CiFT2*, *LFY* and *AP1,* and the flowering inhibitors *FLC, SVP* and *TEM1* also showed alternate bearing in ON and OFF trees, since they were strongly affected by the fruit (**Fig. 3.3**). In Year 1 just before bud sprouting (January), *CiFT2* (**Fig. 3.3 A**), *LFY* and *AP1* (**Fig. 3.3 A**, bar chart) relative expression was significantly higher in OFF buds than in ON buds. As a result, OFF trees sprouted and flowered profusely in spring (Year 2) (becoming ON) whereas ON trees sprouted less and did not flower (becoming OFF). Later, *CiFT2* showed significant differences in leaves between ON and OFF trees from November onwards. In OFF-tree leaves, *CiFT2* expression was up-regulated, becoming more than 15-fold higher than that of ON-tree leaves in January, and remaining almost constant up to late in February. *CiFT2* in ON-tree leaves did not vary significantly during the study period (**Fig. 3.3 A**). On the contrary, the fruit stimulated the *FLC* gene expression in ON-tree leaves from October to the end of February (**Fig. 3.3 B**). But what is more important, the expression of *FLC* in buds in January, and in leaves in May did not differ significantly between ON and OFF trees. This suggests a kind of mechanism that inhibits flower induction in the adult leaf (and CiFT2 protein is not transferred to the meristem), but allows the meristem to restart and develop new leaves without the inhibitory signal. Afterwards, when leaves and fruits

reach the adult stage, *FLC* is again expressed in ON-tree leaves. The *SVP* gene, which directly inhibits *FT* together with *FLC*, showed a similar trend to that of *FLC*. In Year 2 the *SVP* relative expression was higher in ON than OFF leaves, but differences were only significant in December (**Fig. 3.3 C**). Finally, *TEM1* relative expression was increased 80-fold in ON-tree mature leaves in January (**Fig. 3.3 D**).

In the 'Moncada' mandarin, alternate bearing is a natural process. A defruiting treatment in ON trees in year 1 only increased flowering in year 2. But the third year, trees began again the ON-OFF cycle (**Fig. 3.1 E and F**). Therefore, an endogenous/genetic mechanism may regulate the process.

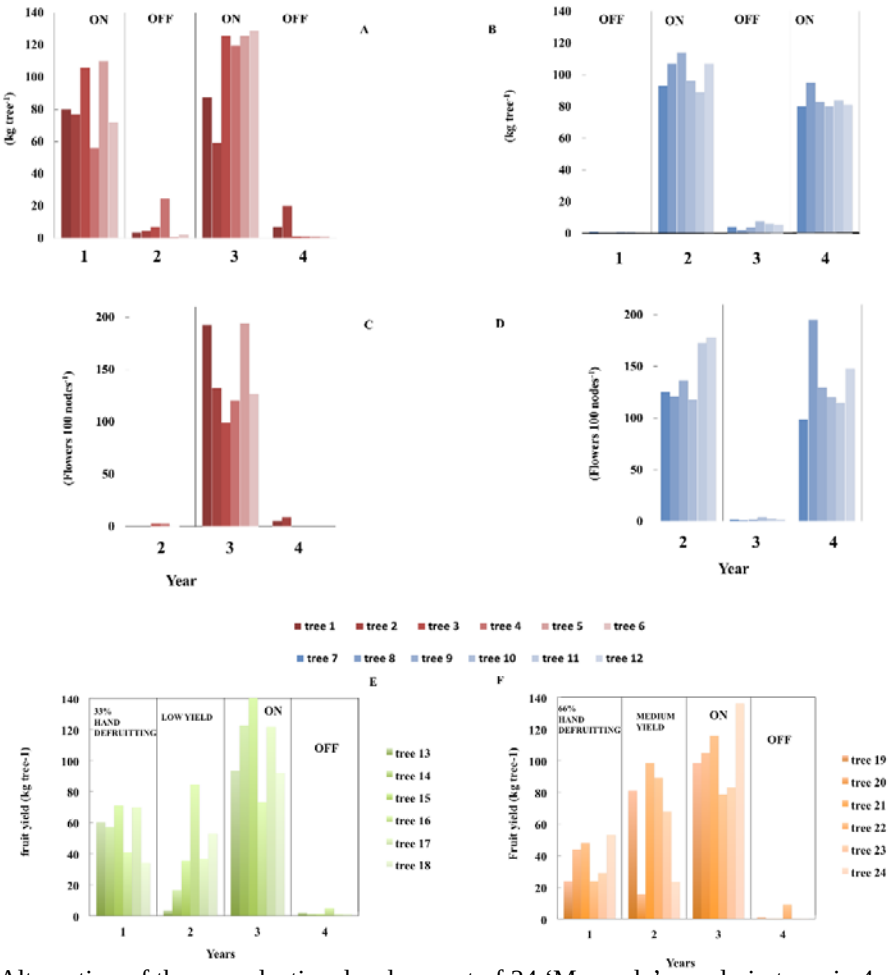


Figure 3.1 Alternation of the reproductive development of 24 'Moncada' mandarin trees in 4 years. Trees 1-6 (red, **A**: fruiting; **C**: flowering) and 7-12 (blue, **B**: fruiting; **D**: flowering) were ON (with fruits) and OFF (without fruits), respectively, in the first year (**A** and **B**). Trees 13-18 (green, **E**) and 19-24 (orange, **F**) were ON trees in year 1 defruited by hand (in summer) up to 33% and 66%, respectively. Year 3 were ON again as it did the red trees.

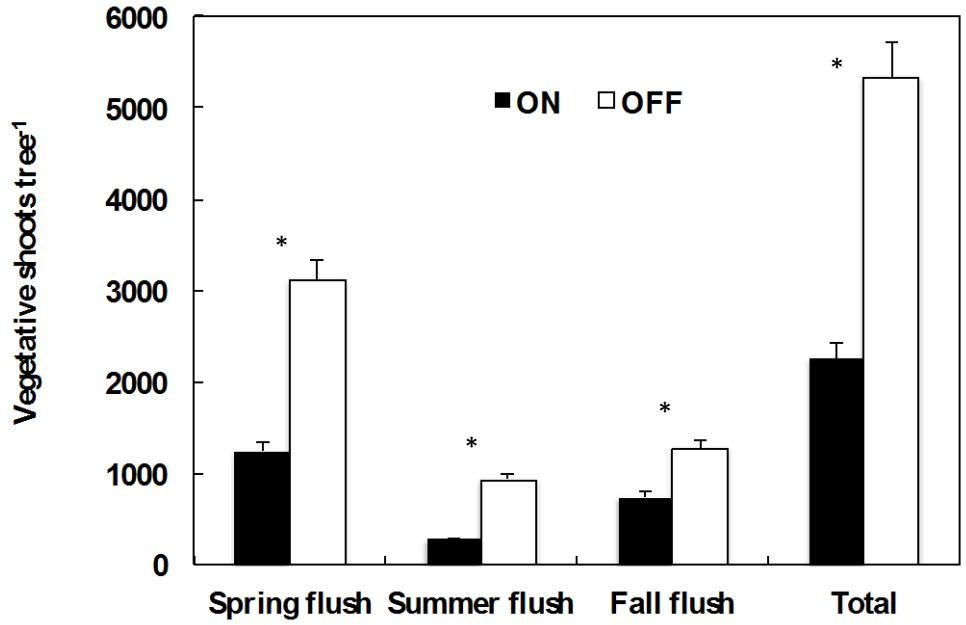


Figure 3.2 Vegetative growth in 'Moncada' mandarin ON (black) and OFF (white) trees. Data are means ± ES of 6 trees. *: indicate significant differences ($p \leq 0.05$).

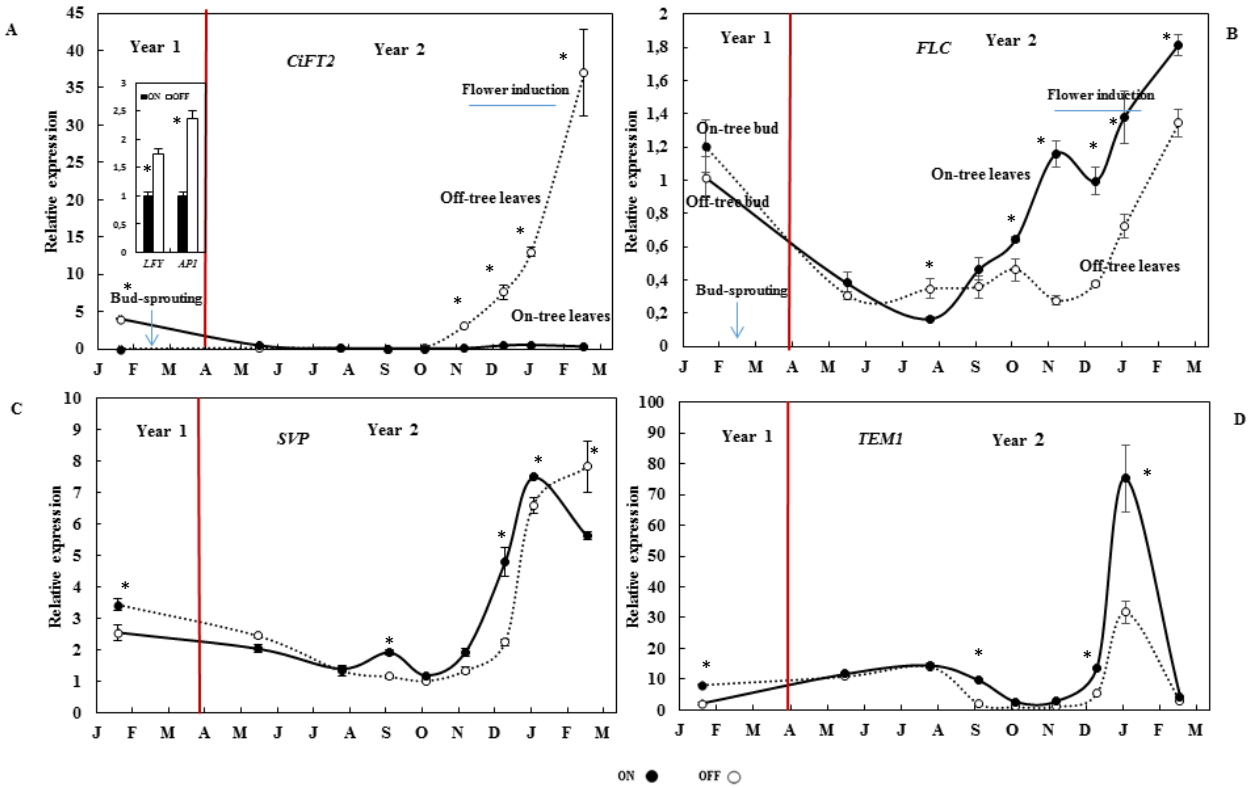


Figure 3.3 *CiFT2*, *LFY* and *AP1* (**A**), and *FLC* (**B**), *SVP* (**C**) and *TEM1* (**D**) relative expression in 'Moncada' mandarin buds (Year 1) and leaves (Year 2) from January to February of the next year. Data are means ± ES of 3 qRT-PCR replicates. *: indicate significant differences ($p \leq 0.05$).

Results suggest that flowering promoters might be up-regulated by exogenous cues (i.e. low temperature) whereas flowering inhibitors might be up-regulated by endogenous cues. The objective of this chapter is to determine the existence of DNA methylation marks that control expression of the genes that negatively regulate flowering (*FLC*, *SVP* and *TEM1*). Methylation of cytosine in DNA is a common epigenetic signaling tool that cells use to lock genes in the "OFF" position. Cytosine methylation occurs in three sequence contexts, cytosine-guanine (CG), the most abundant, but also in CHG and CHH (where H is any nucleotide except G). As a general rule, the more DNA methylation found near the promoter region the more gene silencing. Depending on the DNA methylation profile gene expression can be up-regulated or otherwise blocked. Histone methylation is another epigenetic mechanism that regulates gene expression. Several studies have indicated that DNA methylation and histone methylation at certain positions are connected.

3.2 DNA methylation profiles

3.2.1 During the flower induction period

3.2.1.1 Flowering promoters

DNA-methylation was studied in leaves sampled on November 30th (flower induction period), when a clear difference was found in *CiFT2*, *FLC* and *SVP* gene expression between ON and OFF trees (**Fig. 3.3**).

DNA methylation was studied in both *CiFT1* and *CiFT2*. In the *CiFT1* gene sequence, three regions were found to present CG sites: promoter-intron (in 2 positions, 1 and 2), intron (in 3 positions, 3, 4 and 5) and exon (in 6 positions, 6-11) (**Fig. 3.4 A**). However, no cytosine was found to be metilated in these CGs in ON and OFF leaves (**Fig. 3.4 B and C**). Neither CHG nor CHH were found to be methylated in the *CiFT1* gene. Thus, they were not represented in **Fig. 3.4 A**. On the other hand, only the promoter region in the *CiFT2* gene was studied. This region presented 7 CG sites (**Fig. 3.4 D**). Only the first CG showed cytosine methylation in 4 (out of 10) analyzed sequences of the ON leaves (**Fig. 3.4 E**). This scarce DNA methylation profile suggests no relationship with *CiFT2* silencing in ON-tree leaves. The *CiFT2* gene showed no DNA methylation in

OFF-tree leaves (**Fig. 3.4 F**). Results suggest that differences in *CiFT2* gene expression between ON and OFF trees (**Fig. 3.3 A**) are not regulated by DNA methylation.

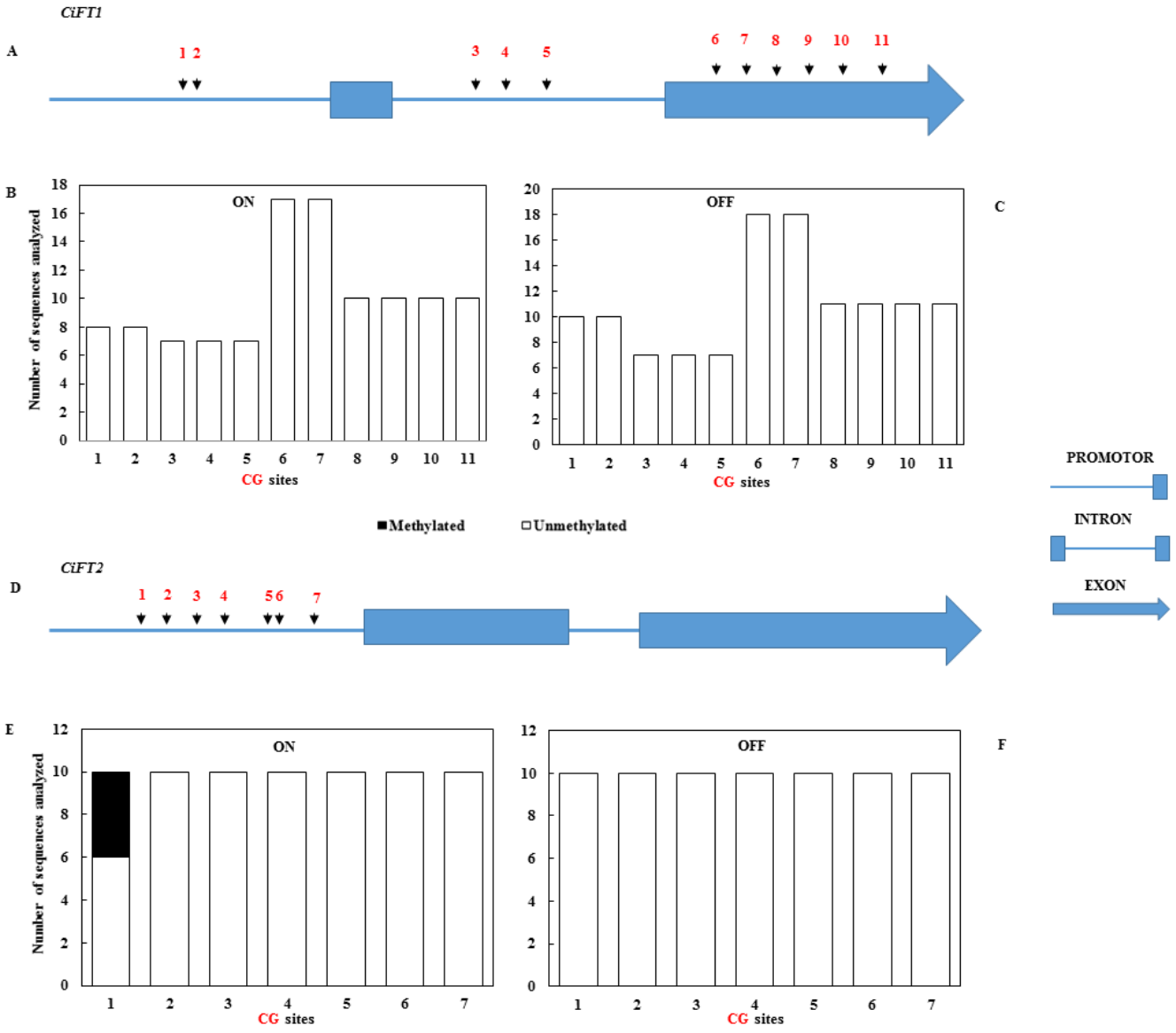


Figure 3.4 DNA methylation profiles of *CiFT1* and *CiFT2* in ON and OFF trees in the flower induction period (November). **A and D**: Scheme of the analyzed zone. Red numbers indicate CG sites in the sequence of the analyzed gene. **B, C, E and F**: results of the bisulphite sequencing analysis of at least 7 clones per tree. White bars indicate no cytosine methylation whereas black bars indicate cytosine methylation. DNA was collected from ON and OFF leaves during the floral bud inductive period (November) of 'Moncada' mandarin.

3.2.1.2 Flowering inhibitors

The *FLC* gene presented 24 CG sites in the promoter region and 10 CG sites in the intron (**Fig. 3.5 A**). After the bisulfite treatment, 4 CHH sites (positions 20, 30, 32 and 35) showed differences in cytosine methylation between ON and OFF leaves and thus were included in **Figure 3.5**. In the promoter region, CG sites showed no methylation in either ON or OFF trees, and only the position 20 (CHH) showed partial methylation (4 out of 10 clones) in OFF leaves (**Fig. 3.5 B and C**). On the other hand, ON-tree leaves showed more cytosine methylation in the intron than OFF-tree leaves. ON-tree leaves showed methylation changes compared to OFF-tree leaves in the positions 27 (100% vs 33% CG), 29 (0% vs 66% CG), 30 (33% vs 0% CHH), 31 (66% vs 33% CG), 32 (0% vs 33% CHH), 35 (100% vs 0% CHH) and 37 (100% vs 0% CG). These significant differences in the methylation profile may explain the significant difference found in the *FLC* gene expression in November between ON and OFF trees (**Fig. 3.3 B**).

The *SVP* and *TEM1* methylation profiles did not show significant differences between the ON-tree and OFF-tree leaves. While *SVP* showed no methylation in either ON- or OFF-tree leaves (**Fig. 3.5 E and F**), *TEM1* showed all the CG sites methylated (**Fig. 3.5 H and I**), which were found in the promoter region (**Fig. 3.5 G**). Epigenetic regulation of the *TEM1* gene cannot be discarded since its relative gene expression did not differ between ON and OFF trees in November (**Fig. 3.3 D**). However, the temporal pattern of *TEM1* gene expression in relation to *CiFT2* suggests a secondary role of this gene negatively regulating flowering in citrus. On the other hand, *FLC* might play a key role regulating the process. To better relate the methylation profile of the *FLC* gene with its expression, the DNA-methylation study was repeated in young leaves sampled in May (before the flower induction period), when *FLC* gene expression did not differ between ON and OFF trees (**Fig. 3.3 B**).

3.2.2 Before the flower induction period

3.2.2.1 *Flowering Locus C*

Before the flower induction period (May), the *FLC* methylation profile of ON- and OFF-tree leaves showed high similarity. Almost all the same sites showed DNA methylation. Only the position 34 showed an absolute difference (0% ON vs 100% OFF

CG), and the positions 29 (100% vs 54.5% CG) and 31 (18.2% vs 100% CG) showed partial methylation (**Fig. 3.6**).

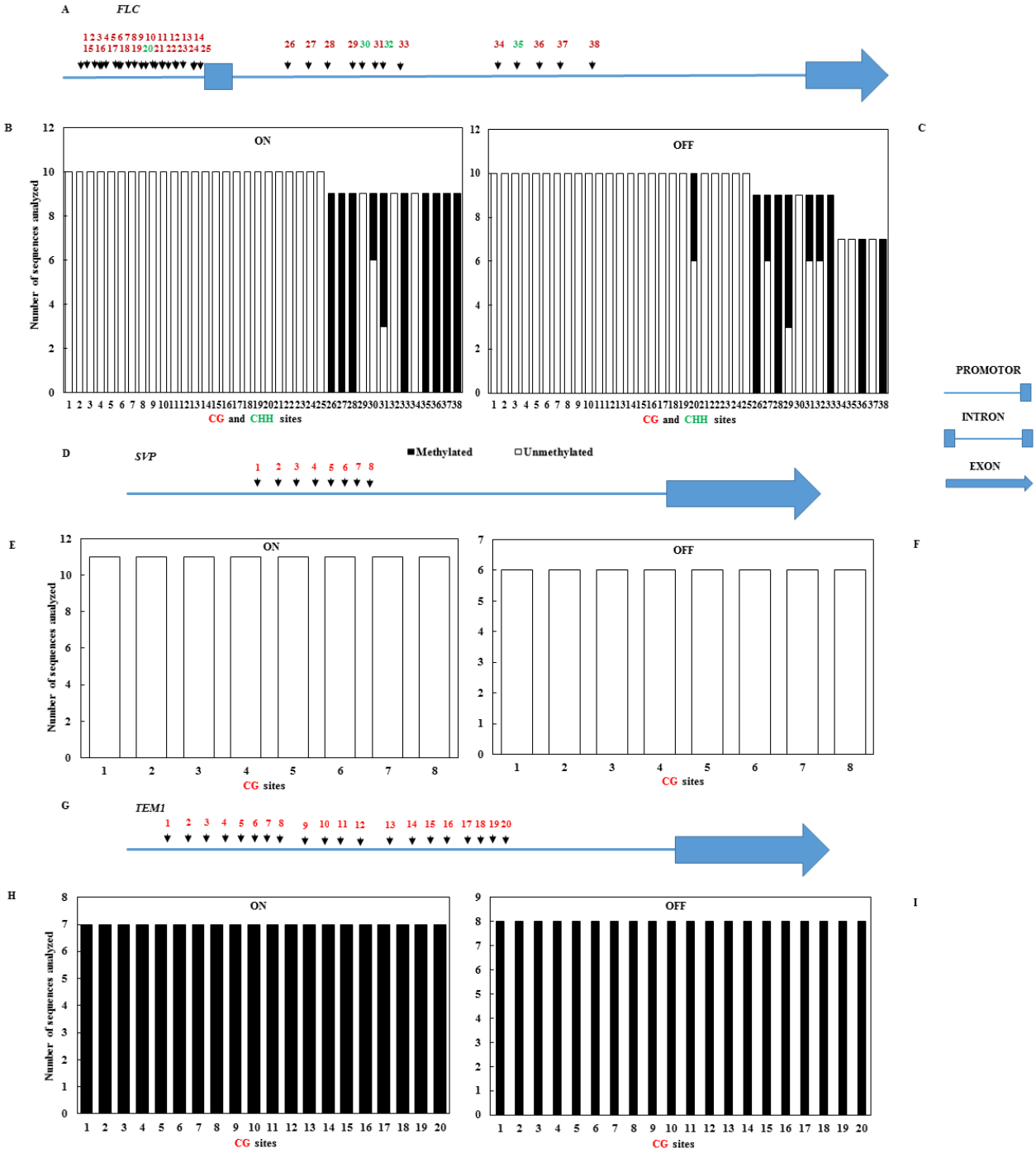


Figure 3.5 DNA methylation profiles of *FLC*, *SVP* and *TEM1*. Scheme of zone analyzed. Bisulphite sequencing of promoter and intron regions was performed on DNA collected from ON and OFF leaves during the floral bud inductive period (November 30) of 'Moncada' mandarin.

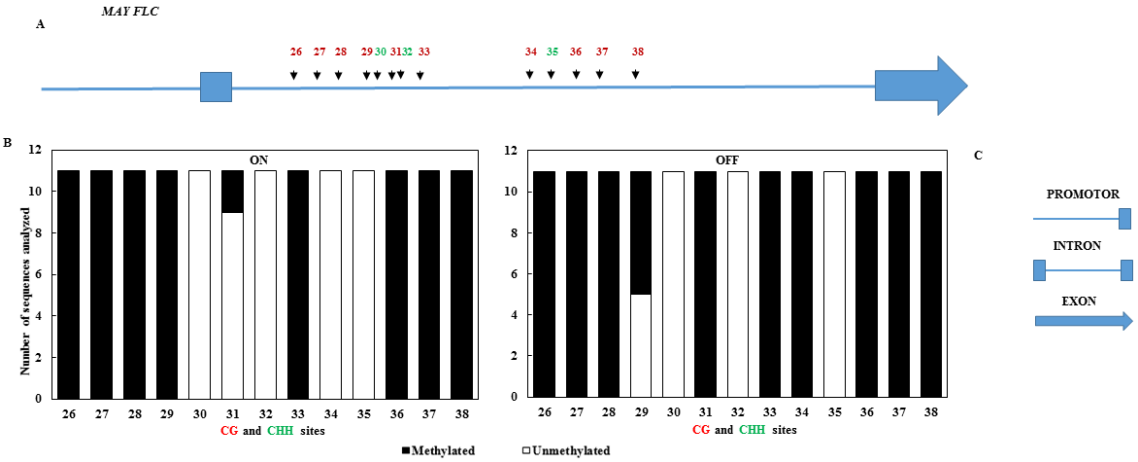


Figure 3.6 DNA methylation profile of *FLC*. Scheme of zone analyzed, and bisulphite sequencing of promoter and intron regions was performed on DNA collected from ON and OFF leaves (May 13) of 'Moncada' mandarin.

It is worth nothing that while in May the *FLC* gene showed DNA methylation in the same positions for ON and OFF leaves (except position 34) (**Fig. 3.7**), and also the same relative expression (**Fig. 3.3**), in November, both DNA methylation and relative expression of the *FLC* gene differed significantly between ON-tree and OFF-tree leaves (**Figs. 3.7 and 3.3**). In November, DNA methylation increased in ON-tree leaves compared to May, whereas it decreased in OFF-tree leaves (**Fig. 3.7**). This result raises the question of which methyltransferases modify their activity in ON and OFF leaves between May and November coinciding with the up-regulation of *FLC* gene expression.

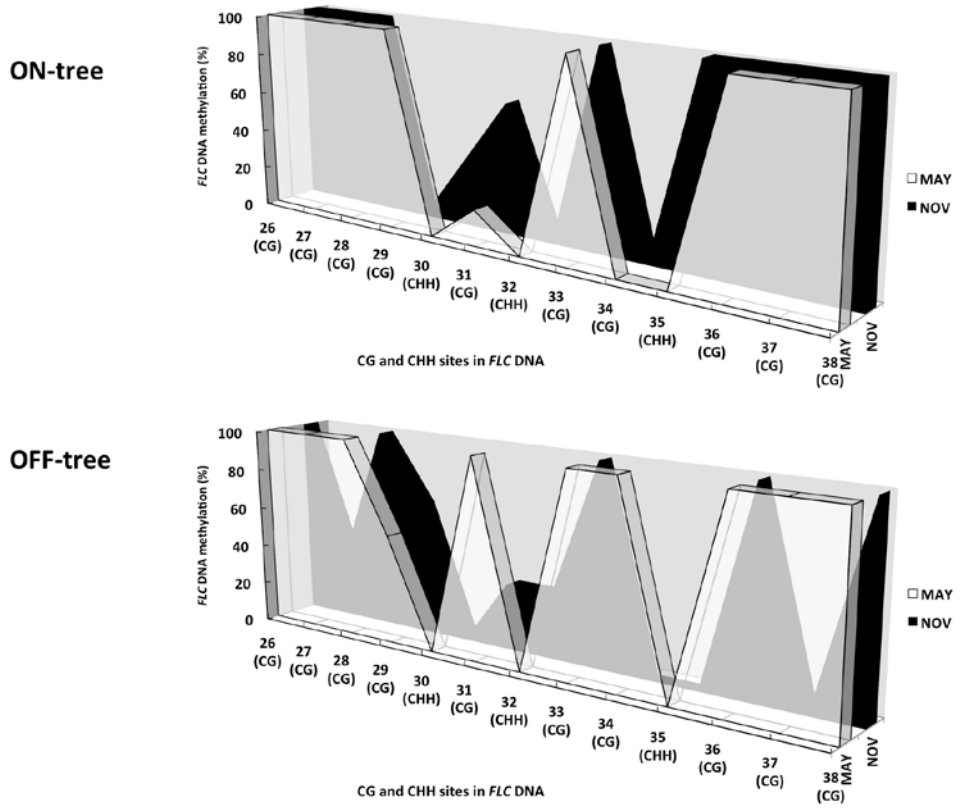


Figure 3.7 Schematic representation of the DNA methylated sites in the *FLC* gene in May (white) and November (black) in ON and OFF trees of the 'Moncada' mandarin.

3.3 Time-course of methyltransferase gene expression

Several methyltransferases have the ability to promote or repress *FLC* gene expression by directly methylating *FLC* DNA and indirectly, by inducing changes in the histones.

3.3.1 Promoters of *FLC* expression

Among the promoters, the methyltransferase *ELF8* directly methylates *FLC* DNA whereas *12ATX* and *7ATX* are involved in histone methylation.

The relative expression of *ELF8* differed significantly in ON and OFF leaves in September and during the flower induction period (December) (1.2 ON vs 0.4 OFF and 2.9 ON vs 0.6 OFF, respectively). Differences were maintained between ON and OFF

trees until the end of February (**Fig. 3.6 A**). The relative expression of *12ATX* was significantly higher in buds from ON trees compared to buds from OFF trees (Year 1). After bud sprouting (Year 2), the *12ATX* expression decreased and it did not differ between ON and OFF leaves until December, when it significantly increased in ON-tree leaves (14-fold). Significant differences were not found in January or in February between ON and OFF leaves (**Fig. 3.6 B**). Similar results were found for *7ATX* gene expression (**Fig. 3.6 C**).

These results are in concordance with the higher *FLC* relative expression found in ON-tree leaves compared to that of OFF-tree leaves during the flower induction period (**Fig. 3.3 B**).

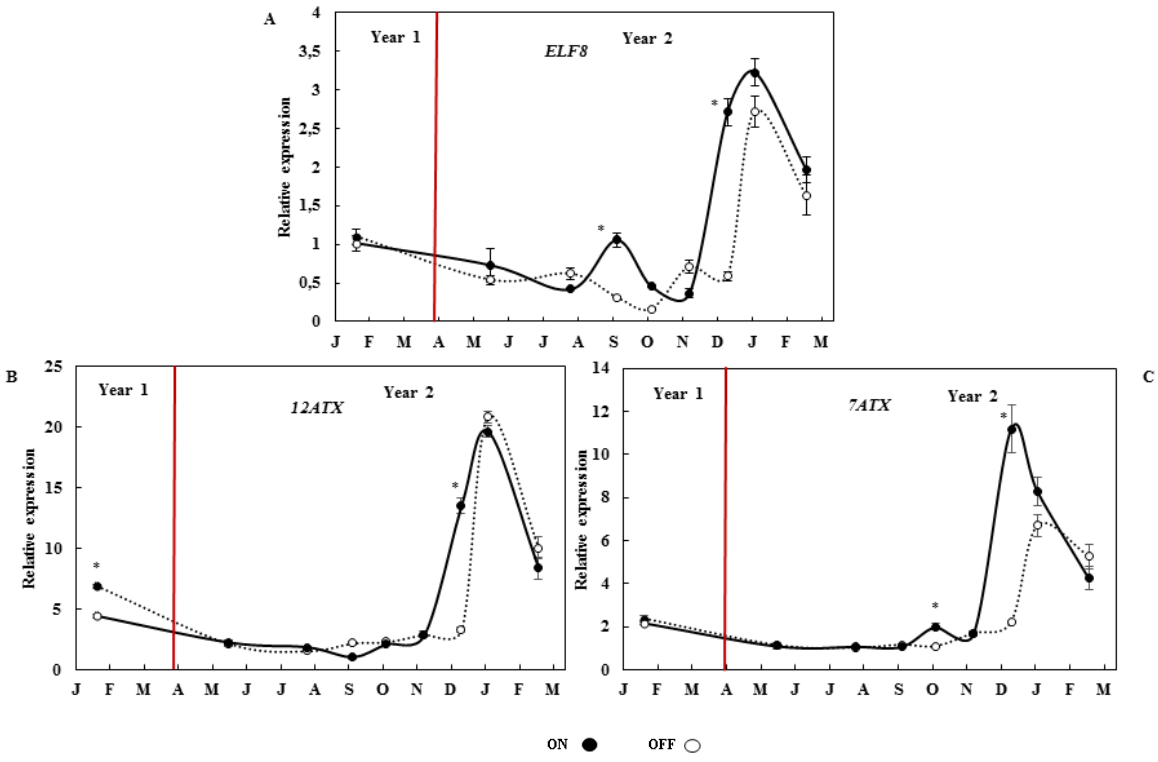


Figure 3.6 *ELF8 (***A***) 12ATX* **(B)** and *7ATX (***C***)* relative expression in 'Moncada' mandarin buds (Year 1) and leaves (Year 2) from January to February of the next year. Data are means ± ES of 3 qRT-PCR replicates. *: indicate significant differences ($p \leq 0.05$).

3.3.2 Inhibitors of *FLC* expression

Among the inhibitors, *SKB1* directly methylates *FLC* DNA whereas *5ATX* is involved in histone methylation. *SKB1* relative expression was significantly upregulated in OFF-tree leaves at the beginning of the flower induction period (November); it differed significantly (2.6-fold) from the *SKB1* expression found in ON-tree leaves (**Fig. 3.7 A**). On the other hand, the *5ATX* gene expression was significantly higher in ON-tree leaves in December and January and in ON-tree buds in January compared to the OFF-trees (**Fig. 3.7 B**).

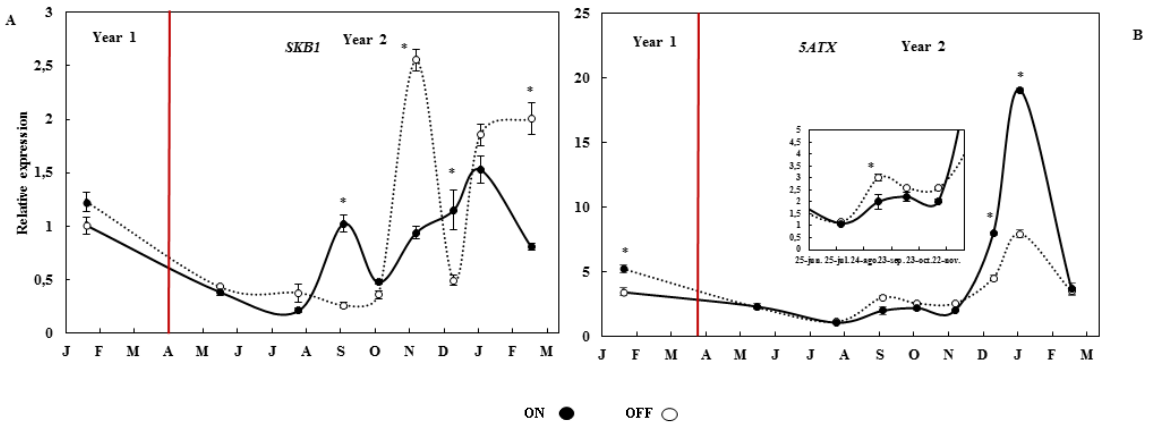


Figure 3.7 *SKB1(A)* and *5ATX (B)* relative expression in 'Moncada' mandarin buds (Year 1) and leaves (Year 2) from January to February of the next year. Data are means ± ES of 3 qRT-PCR replicates. *: indicate significant differences ($p \leq 0.05$).

Results suggest a relationship between DNA methylation (and histone methylation) and *FLC* expression in citrus. To further characterize this relationship, the effect of 5-azazytidine on *FLC* and *CiFT2* gene expression was studied. Azazytidine is a chemical analogue of the cytosine nucleoside that at low doses inhibits DNA methyltransferase causing hypomethylation of DNA.

The treatment significantly increased *FLC* expression from 10 to 50 hours after treatment. Conversely, the *CiFT2* gene expression was significantly reduced, probably due to the increase in *FLC* (**Fig. 3.8**). Results indicate a relationship between DNA methylation and *FLC* expression. However, the effect observed was unexpected: in the ON-OFF system the more DNA methylation the more *FLC* expression. But 5-azazytidine, which causes hypomethylation, also increased *FLC* expression, suggesting a complex relationship between methylation, *FLC* expression and flowering.

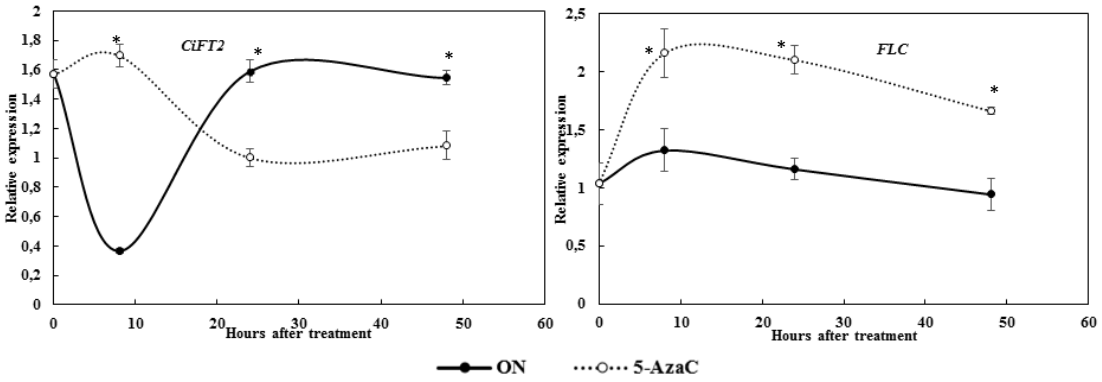


Figure 3.8 Effect of 5-Azacytidine (5-AzaC, 350µM) applied at floral bud inductive period (November 25) on the time course of *CiFT2* and *FLC* relative expression in the leaves of the single flowered leafy shoots of 'Afourer' mandarin. Treatment was applied as a foliar spray. Data are means ± ES of 3 qRT-PCR replicates. *: indicate significant differences ($p \leq 0.05$).

Another important question that requires confirmation is the relationship between fruit, *FLC* methylation and *FLC* expression. Significant differences in the methylated positions of the *FLC* gene were found for ON and OFF trees when *FLC* expression was significantly different (November); moreover, DNA methylated positions did not differ when *FLC* expression was the same in ON and OFF trees. However, this relationship does not imply causality; therefore, further experiments were carried out to demonstrate the direct effect of fruit removal upon *FLC* methylation and gene expression.

3.4 Effect of defruiting on flowering, methyltransferases and flowering gene expression and FLC-DNA methylation

3.4.1 Effect of defruiting on flowering

The effect of defruiting on flowering intensity is shown in **Fig. 3.9**. As expected, ON trees had the lowest number of flowers in spring (6 flowers/100 nodes) in comparison with OFF trees, which had the highest number of flowers (100 flowers/100 nodes). Defruited trees (in August) flowered in spring as did OFF trees (90 flowers/100 nodes), without differing significantly (**Fig. 3.9**).

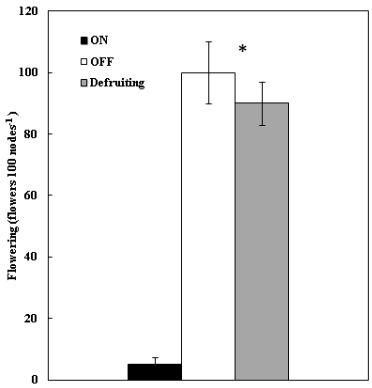


Figure 3.9 Effect of defruiting on flowering intensity of 'Afourer' mandarin branches. Fruits were removed in August. ON: with fruits; OFF: without fruits. Data are the means of 3 trees ± standard error. *: indicate significant differences ($p \leq 0.05$).

3.4.2 Effect of defruiting on flowering gene and methyltransferases relative expression

3.4.2.1 Flowering genes

As expected, defruiting significantly increased the *CiFT2* relative expression and reduced the *FLC* gene expression up to the OFF-tree level. ON trees showed significantly lower *CiFT2* and higher *FLC* gene expressions (**Fig. 3.10 A and B**). On the other hand, defruiting did not significantly modify SVP or TEM1 gene expression (**Fig. 3.10 C and D**).

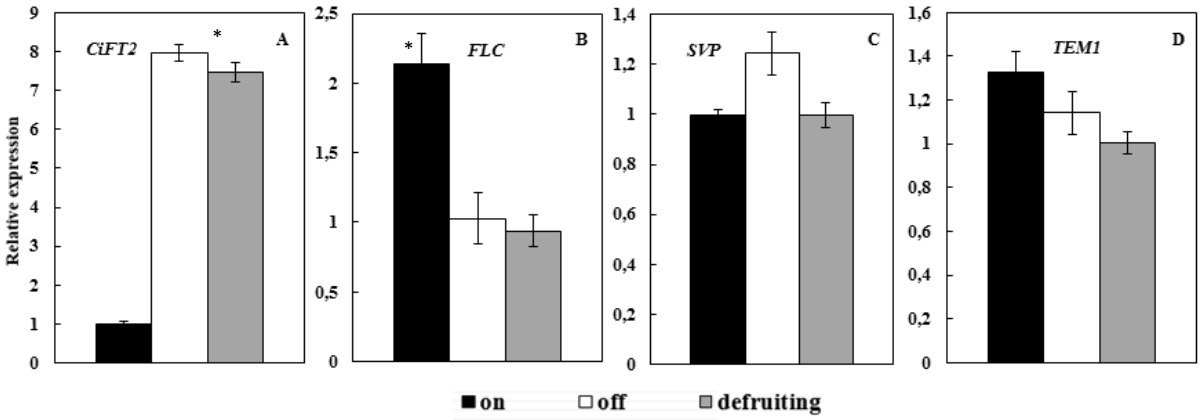


Figure 3.10 Effect of fruit and defruiting on *CiFT2* (**A**), *FLC* (**B**), *SVP* (**C**) and *TEM1*(**D**) relative expression in 'Afourer' mandarin leaves at floral bud inductive period (November 25). Fruits were removed in August. ON (black): with fruits; OFF (white): without fruits. Data are means ± ES of 3 qRT-PCR replicates. *: indicate significant differences ($p \leq 0.05$).

3.4.2.2 Methylation genes

Compared to the ON trees defruited trees had a significant reduction in (6-fold) the expression of the *FLC* promoters *12ATX* and *7ATX*, and it did not significantly differ from that of the OFF trees. No significant differences were found in the *ELF8* gene expression (**Fig. 3.11 A, B and C**). Similarly, defruiting did not modify the relative expression of the *FLC* inhibitors *SKB1* and *5ATX*, which showed the highest value in OFF trees (34x compared to ON trees) (**Fig. 3.11 D and E**).

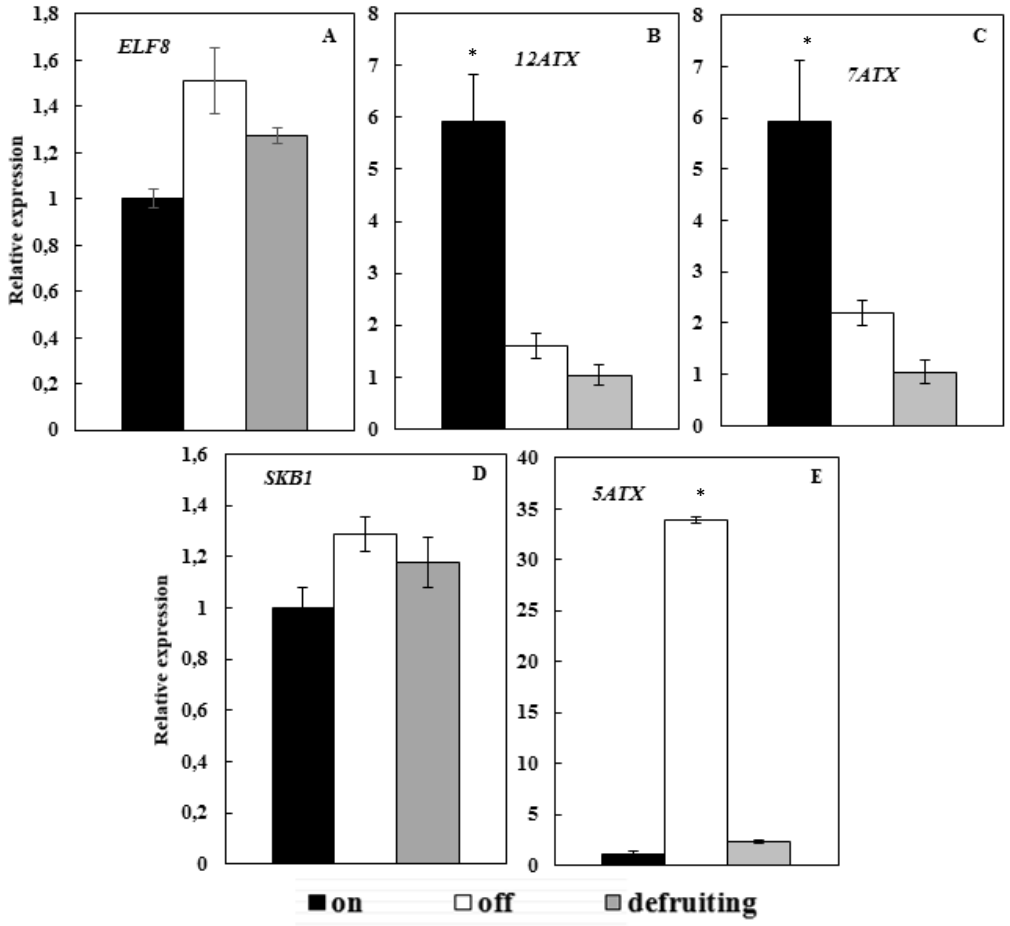


Figure 3.11 Effect of fruit and defruiting on *ELF8 (***A***), 12ATX (***B***), 7ATX (***C***), SKB1 (***D***)* and *5ATX (***E***)* relative expression in 'Afourer' mandarin leaves at floral bud inductive period (November 25). Fruits were removed in August. ON (black): with fruits; OFF (white): without fruits. Data are means ± ES of 3 qRT-PCR replicates. *: indicate significant differences ($p \leq 0.05$).

3.4.3 DNA methylation profile

To analyze the effect of defruiting on *FLC* methylation, the intron region was studied (**Fig. 3.12 A**). *FLC* DNA profile was analyzed in November, i.e during the flower induction period. Defruited and OFF trees showed DNA methylation in the same sites except for position 29, which was partially methylated in OFF trees (45.5% OFF vs 0% DEF CG). On the other hand, ON tree methylation profiles significantly differed from Defruited and OFF trees in the positions 26a (45.5% ON vs 0% OFF and DEF CHH), 27a (100% ON vs 0% OFF and DEF CHH), 29 (100% ON vs 45.5% OFF and 0% DEF CG), and 31 (0% ON vs 100% OFF and 45.5% DEF CG) (**Fig. 3.12**). The differences and similarities between these profiles can be observed in **Fig. 3.13.** Results suggest a clear relationship between fruit, *FLC* methylation, *FLC* expression, *CiFT2* expression and, therefore, flowering.

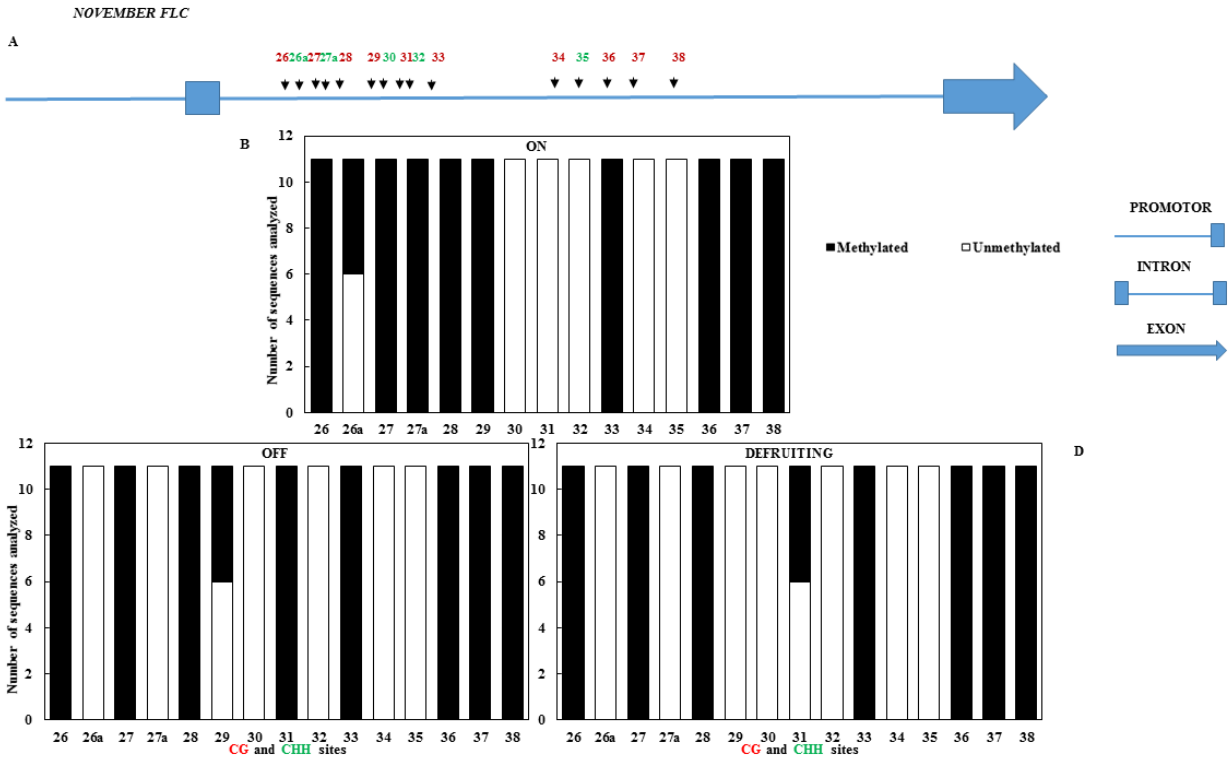


Figure 3.12 DNA methylation profile of *FLC*. Scheme of zone analyzed. Bisulphite sequencing of promoter and intron regions was performed on DNA collected from leaves at floral bud inductive period (November 25) of 'Afourer' mandarin.

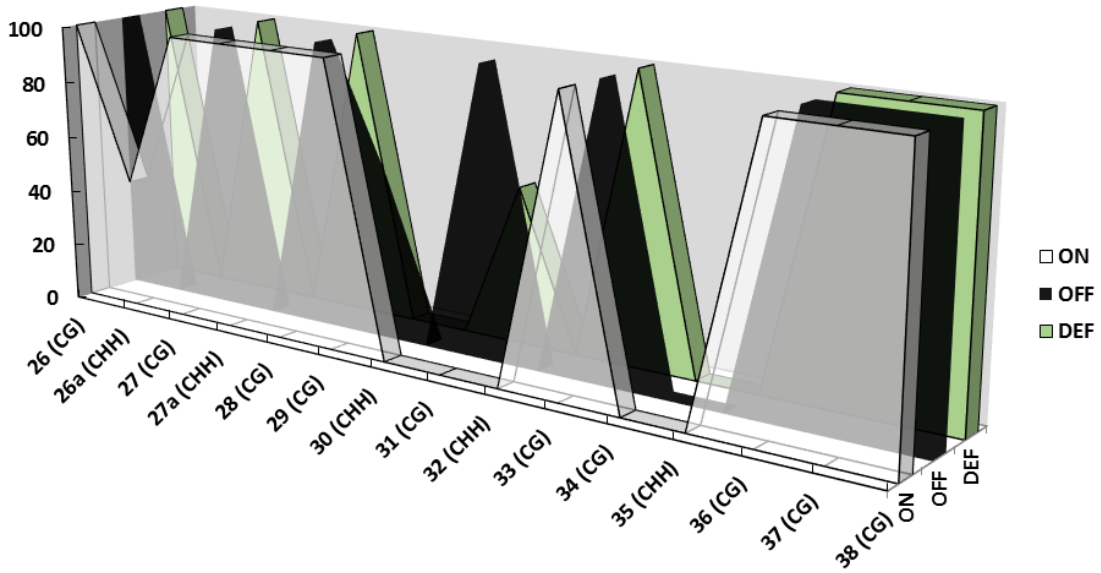


Figure 3.13 Schematic representation of the DNA methylated sites in the *FLC* gene in ON (white), OFF (black) and Defruiting (green) trees of the 'Afourer' mandarin.

Discussion

Alternate bearing in adult trees has been associated primarily with the presence of fruit (Verreynne and Lovatt, 2009; Martínez-Fuentes *et al.*, 2010). In most tree species, bud development and flower induction overlap with the end of fruit development and, according to the literature, it is assumed that fruit alters both flower induction and floral bud differentiation, and thus inhibits flowering (Valiente and Albrigo, 2004). The mechanisms regulating this process are not fully understood. The traditional explanation points to the GAs produced by the fruit (in developing seeds or other tissues), which are supposed to be exported and inhibit flower bud development. This Ph.D research suggests that the fruit-governed hormonal regulation of flower bud induction is coordinated with a molecular regulatory system that controls the expression of flowering inhibitor genes (i.e. *FLC* gene).

This research demonstrats that every bud beside a fruit does not have the ability to produce a flower or inflorescence, and needs to *restart* vegetative growth developing new buds that will flower or not (**Figs. 1.1 and 1.14**). These new buds are usually produced during the fruit growth stage (i.e. summer and fall flush, **Fig. 3.2**), and they will flower in spring only if they are *away* from *fruit-developing signals* (**Fig. 1.14**). This was indirectly demonstrated with the girdling experiment (**Fig. 1.19**) and it is what is usually observed in medium-yield trees (**Figs. 1.12 and 1.14**). In ON-trees, the new buds from summer and fall flushes (**Fig. 3.2**) are also under the influence of *fruit-developing signals*, and do not flower in spring (**Figs. 1.1, 1.12 and 3.1**).

Conversely, in OFF-trees, a considerable number of new buds are developed in vegetative shoots from spring, summer and fall flushes (**Fig. 3.2**), and almost all (80%) are induced to flower (**Fig. 1.1**). The presence of flowering promoters, produced in the leaves, is needed for flowering. In fact, ON-tree buds flowered when they were partially isolated from the fruit influence (girdling experiment, **Fig. 1.19**), but they did not flower when they were totally isolated from the fruit and the tree (*in vitro* experiment). Further, OFF buds induced to flower lost their ability when cultured *in vitro*.

Particularly, in citrus grown in Mediterranean climates flower induction occurs late in autumn (due to low temperatures), whereas differentiation occurs in late winter or spring when temperatures rise (Agustí, 2003). The regulatory role of the *FLOWERING LOCUS T (FT)* gene has been identified in numerous species (Bangerth, 2009; Wilkie *et al.*, 2008) including *Citrus* (Endo *et al.*, 2005; Nishikawa *et al.*, 2007, 2009; Muñoz-Fambuena *et al.*, 2011, 2012a, 2012b). Under floral bud inductive conditions (low temperatures and low fruit loads), Ci*FT* expression starts in October

and November, for 'Moncada' mandarin (Muñoz-Fambuena *et al.*, 2011) and 'Satsuma' mandarin (Nishikawa *et al.*, 2007), respectively. Results for the 'Moncada' mandarin are confirmed here (for the *CiFT2* paralog), but also for other species such as 'Salustiana' sweet orange and 'Afourer' hybrid mandarin.

In this Ph.D. Thesis the *FLC* gene has been characterized in *Citrus clementina* and its function in the induction process. The different studies in *Arabidopsis thaliana* have related this gene with the inhibition of the flowering (Blazquez *et al.*, 2011), and in this research the same function has been confirmed in *Citrus*.

Fruit-derived signals (GAs and ABA) overlap with CiFT2 and FLC gene expression

Martínez-Fuentes *et al.* (2010) demonstrated that the fruit exerts its inhibitory effect on flowering from the time it is close to reaching its maximum weight, namely 90% of its final size (November, in 'Valencia' sweet orange), and Muñoz-Fambuena *et al.* (2011) correlated this with the expression of the *CiFT* gene. At this stage, fruit cell enlargement is almost complete and fruit begins the ripening stage (Agustí, 2003). During the cell enlargement stage, the fruit produces auxin and GAs in the peel and the pulp (≈ 7 ng g^{-1} IAA and 2 ng g^{-1} GA_4, **Fig. 1.18**). Auxins are basipetally transported through the peduncle (Bangerth, 2000). Several experiments related to fruit abscission demonstrated that when the fruit reaches its final size, the basipetal auxin flow through the peduncle is progressively diminished (Estornell *et al*., 2013).

On the other hand, Alquezar *et al*. (2008, 2009) showed that the peel (mainly) and the pulp are rich in chloroplasts that are converted into chromoplasts. This process is inversely related to GAs (Gambetta *et al.*, 2012), and as a consequence of carotenoid synthesis, ABA is also produced (Rodrigo *et al*., 2003). In 'Moncada' mandarin ON-trees, the start of chlorophyll degradation in the exocarp (October) coincided with a decrease in the GAs content (**Figs. 1.4 and 1.5**), whereas the increase in the yellow color (b, Hunter, **Fig. 1.4**) during degreening coincided with a significant increase in the ABA content (**Fig. 2.6**). In *Citrus*, pigments that produce yellow coloring are violaxantin and neoxantin, which are the precursors of the ABA synthesis (Alquezar *et al.*, 2008; Davies, 2010). At the same time 1) both ABA and GAs (GA_{20} and GA_1) significantly increased in the leaves adjacent to the fruits (**Figs. 1.5 and 2.6**) whereas they decreased or were not modified in the OFF-tree leaves (**Figs 1.6 and 2.6**); 2) *FLC*

gene expression was upregulated in ON-tree leaves whereas *CiFT2* gene expression was upregulated in OFF-tree leaves.

The increase in the ABA content coincides with previous results reported by Shalom *et al.* (2014). However, this increase is not produced by the endogenous synthesis in buds as Shalom *et al.* (2014) indicated. Since ABA transport is produced in both basipetal and acropetal directions, it is possible that this increase in buds and leaves is due to color change of the adjacent fruits. In *Arabidopsis*, ABA inhibits flowering through the activation of *FLC* expression as indicated by Wang *et al.* (2013). In fact, the mutation of the transcription factor *ABI5* (ABSCISIC ACID INSENSITIVE MUTANT 5) promotes flowering, while the overexpression of *ABI5* activates *FLC* expression. When ABA concentration increases ABI5 increases too, and, at the same time, these activate the *FLC* relative expression inhibiting flowering. In the 'Moncada' mandarin, the highest increase in *FLC* expression (November) was not followed by a high content of ABA in leaves. Thus, more investigation is needed to clarify the effect of fruit on the relationship between ABA and *FLC* upregulation in citrus.

Regarding GAs, Bangerth (2009) advocated considering endogenous GAs as ''directed'' long-distance signals to inhibit flower induction in trees, although GA treatments inhibit flowering in most fruit tree species. Specifically, Bangerth (2009) stated "it would be difficult to assume transport from a fruit, a strong sink, to the apical or axillary bud, a weak sink". Nonetheless, Prang *et al.* (1998) demonstrated an export of GAs from apple fruit as well as from shoot tips with a peak occurring at the time presumed flower induction occurs. Moreover, in citrus, Gambetta *et al.* (2012) highlighted the need for GA export to initiate change color. Since the fruit sink strength is significantly diminished at the ripening stage, results in the 'Moncada' mandarin might be taken in consideration. Further, the fruit-to-adjacent leaves GA transport hypothesis, at the ripening stage, is reinforced by 1) the reduced *GA20ox* and *GA3ox* activity found in the leaves (**Fig. 1.7**) when GA_{20} and GA_1 are significantly higher (**Fig. 1.5**); and 2) the absence of GA_1 and the catabolites GA_8 and GA_{29} in the fruit (**Fig. 1.5**). Regnault *et al.*, (2015) recently demonstrated that the long-distance GA transport in the plant is manly produced through the precursor GA_{12}. However, these authors do not discard the transport of others precursors apart from GA_{12}. The significant differences in GA biosynthesis and content in ON and OFF trees in September are due to the different developmental stages. Leaves from OFF trees belonging to the summer and fall flushes are young developing leaves, compared to mature leaves from the spring flush in ON

trees, and, therefore, GAs and also IAA concentrations are significantly high (**Fig. 1.8**). GAs and IAA directly regulate cell division and elongation in the leaf (Nelissen *et al.*, 2012).

GA regulation of flowering

Guardiola *et al.* (1982) established two periods of sensibility to GA treatments for flowering inhibition in *Citrus*. These authors suggested that GA might interfere with the *flowering signal* during the first period (i.e. November-December). Moreover, they identified a direct effect of GAs on the bud during the second period of sensibility, coinciding with the onset of morphological differentiation in the flower (i.e. February) (Guardiola *et al.*, 1982; García-Luís *et al.*, 1986). The effect of GA and PBZ reducing and increasing, respectively, *CiFT* expression when applied at the end of November (**Fig. 1.10**) might explain the mechanism in the first period of sensibility. The second period of sensibility was not contemplated in this Ph.D. research. However, in the last experiment, PBZ significantly increased *LFY* expression (two months after the treatment, i.e. February), which is related to flower differentiation. This delayed effect might be due to the high persistence of the PBZ molecule. A future experiment design to elucidate the molecular mechanism of the GAs effect in flower differentiation is needed (GA_3 and PBZ applied to the bud). Nevertheless, some clues might be highlighted. Goldschmidt and Samach (2001) analyzed the inverse relationship between flowering and stem elongation, and compared herbaceous annual plants to trees. In *Arabidopsis*, flowering takes place just after elongation of the inflorescence axis. In fruit trees, an antagonism between flowering and stem elongation is also observed. In *Citrus*, flowers are borne in short generative shoots whereas vegetative shoots are long. GA_3 treatment selectively reduced the number of flowering shoots but not the number of flowers per shoot (**Table 1.1**). Thus, GAs limit the ability of the meristem to produce an inflorescence. Recently, this has also been shown in *Arabidopsis*. GAs have a dual opposite role promoting termination of vegetative development but inhibiting flower formation in the meristem. To overcome this effect, *LFY* induces the expression of GA catabolism genes (Yamaguchi *et al.*, 2014). In transgenic *Citrus* juvenile plants, overexpression of *FLY* produces extremely short plants without thorns that develop a flower in the apical meristem (Peña *et al.*, 2001), and the application of PBZ to adult trees increases *LFY* expression, the number of flowering leafless shoots and the number

of flowers per inflorescence (**Table 1.1**). The relationship between GAs-*LFY*-shoot development in citrus deserves further analysis.

PBZ and other growth retardants have been used successfully to promote flowering in fruit tree species such as mango (Blaikie *et al.*, 2004), pear (Asín *et al.*, 2007) and citrus (Delgado *et al.*, 1986; Harty and Van Staden, 1988; Martínez-Fuentes *et al.*, 2004; Monteiro da Cruz *et al.*, 2008; Ogata *et al.*, 1996). However, it is not possible to use growth retardants to promote flowering in ON-trees (Martínez-Fuentes et *al.*, 2004; Monselise and Goldschmidt, 1982; Monteiro da Cruz *et al.*, 2008). Monselise and Halevy (1964) increased flowering in 'Eureka' lemon by spraying the growth retardants CCC, B-Nine, and BTOA during summer, but the results have never been consistent for sweet orange (Agustí, 1980; Moss, 1970), mandarins (Agustí, 1980), limes (Davenport, 1983) or for lemon lateral buds cultured *in vitro* (Tisserat et al., 1990). PBZ applied to the soil increased flowering in acid lime 'Tahiti' trees subjected to low inductive temperatures (16 ºC), but failed to do so in trees grown under high day/night temperatures (25 ºC/20 ºC) (Monteiro da Cruz *et al.*, 2008). Monselise and Goldschmidt (1982) suggested that these erratic effects might be due to the fact that antagonists of GA synthesis should reach the site of synthesis before GAs are produced, the effect depending, therefore, on the timing and method of treatment. In the experiments described in this Ph.D. thesis, PBZ failed to promote flowering under heavy cropping conditions, in agreement with previous reports (El-Otmani *et al.*, 2004), even when modifying the date of treatment (floral bud inductive period or bud differentiation), the method of treatment (applied to the soil or spraying the canopy) or the dose applied (1, 10 or 15 g tree^{-1}) (**Fig. 1.13**). Martínez-Fuentes *et al.* (2004) suggested that endogenous inhibitors (i.e. GA synthesized in the fruit) prevail over exogenous promoters, which cannot counteract their inhibitory effect. In the experiments described here, increasing the PBZ dose from 1 g tree^{-1} up to 10 g tree^{-1} did not improve the response obtained in either OFF-trees or ON-trees, meaning that 1 g tree^{-1} is enough to promote flowering, in agreement with Monteiro da Cruz *et al.* (2008) who reported a saturating response at 0.8 g tree^{-1}. Moreover, fruit load also nullifies the inductive effects of autumn girdling on flowering (Goldschmidt and Golomb, 1982), which is known to effectively increase flowering in sweet orange, Satsuma mandarin, and hybrids (Agustí *et al.*, 1992; Erner, 1988). The present results also reveal a fruit-load threshold value above which PBZ is ineffective to promote flowering. This threshold value varied in these experiments depending on the cultivar (**Fig. 1.12**).

Therefore, the fruit might switch on other flowering gene inhibitors through other metabolic routes apart from GAs. This hypothesis is also supported by the lack of effect of GA_3 and PBZ on *FLC-like* gene expression applied to medium-yield trees (Fig**. 1.10**). At this point, two questions arise: 1) which metabolic routes are up/down regulated in ON and OFF leaves and buds? and 2) how are flowering gene inhibitors activated?

Fruit upregulates stress responses and oxidoreductase activity in leaves and buds during the flower induction period

Significant changes in the leaves and bud proteome of the 'Moncada' mandarin ON and OFF trees were found. The study was conducted in November, during the onset of low temperature flowering induction. Since the OFF-crop buds flowered in the spring following the sample collection year, and the ON buds hardly flowered at all (Muñoz-Fambuena *et al.*, 2011, 2012), knowing how proteins are expressed in each type of leaves and buds (OFF and ON) allows a better understanding of the relationship between alternated bearing and flowering.

Proteins up-expressed in the OFF samples

Proteins related to primary metabolism

The findings indicate that the starch level was significantly higher in leaves of OFF trees than in leaves of ON trees (**Fig. 2.5**), being these results consistent with the up-expression of GBSS in OFF leaves. The same behavior has been described for other biennial-bearing species. For example, in pistachio, during nut development, various organs of OFF trees began to accumulate greater concentrations of soluble sugars and starch, surpassing the amounts measured in organs of ON trees (Banisabab and Rahemi, 2006). Also, in biennial bearing mango trees, the ON trees had a lower starch content in the shoots than OFF trees, during the floral inductive period (Nakagawa *et al.*, 2012). Since starch accumulation in the *Citrus* shoots seems to parallel flower induction (Yahata *et al.*, 2006), the carbohydrate reserves in leaves in 'Moncada' mandarin OFF trees may act like an active sink (Goldschmidt and Golomb, 1982). Moreover, in olive (*Olea europaea*) another study suggested a strong correlation between flower starch

content and functional pistil development (Reale *et al.*, 2009). However, a regulatory role of the leaf carbohydrates in alternate bearing could not be observed (Martínez-Fuentes *et al.*, 2010; Monerri *et al.*, 2011).

The main enzymes related to photosynthesis were also up-expressed in OFF samples in general. Among them, of particular interest is the RuBisCO large subunit-binding protein subunit beta (spots 300, 301, 306 and 307; **Tables 2.1 and 2.2**) or NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (spots 420 and 437). Both of these are involved in the photosynthetic Calvin cycle (Leegood, 1996). This suggests that the light-independent reactions for photosynthesis are stronger in the OFF samples than the ON samples. Another notable up-expressed protein in the OFF samples related to primary metabolism was 6-phosphogluconate dehydrogenase (6PGD; spot 454). This protein is a key enzyme of the pentose phosphate pathway, a part of the central metabolism. Likewise, 6PGD is able to co-operate between the cytosolic and plastidic oxidative pentose phosphate pathways in the provision of NADPH for biosynthesis (Averill *et al.*, 1998).

Among the proteins up-expressed in the OFF buds, certain proteins relating to the carbohydrate metabolism may be highlighted: class III HD-Zip protein HDZ31 (homeodomain-leucine zipper; spot 91); glyceraldehyde-3-phosphate dehydrogenase (G-3-PD, spots 164 and 519); pyruvate kinase (PK, spots 321 and 339); 6-phosphogluconate dehydrogenase (6-PGD, spots 398, 406 and 427) and fructose-bisphosphate aldolase (spots 571 and 1227). The first protein listed, namely HD-Zip protein HDZ31, is involved in the initiation and function of shoot apical meristems (Robischon *et al.*, 2011). The second protein, namely G-3-PD, is involved in the photosynthetic Calvin cycle (Leegood, 1996) and its increased expression was also noted in the 'Moncada' OFF leaves. Other studies have also demonstrated that the genes related to the Calvin cycle in *Citrus* buds (Murcott mandarin) in May are more highly induced in OFF buds than in ON buds (Shalom *et al.*, 2012). The third protein, namely PK, catalyses the synthesis of pyruvate and ATP from phosphoenolpyruvate and ADP, and represents an important control point for plant glycolysis (Knowles *et al.*, 1998). Grodzinski *et al.* (1999) demonstrated that plants deficient in leaf cytosolic PK cultivated under low light intensity exhibit delayed shoot and flower development as compared to plants with wild-type levels of PKc; this deficiency seems to jeopardize the ability of the PK leaf tissue to mobilize carbohydrate reserves at night. Accordingly, the lower PK expression in ON buds may partly contribute to their reduced flowering in the

following spring as compared to the OFF 'Moncada' buds. The fourth protein, 6-PGD, is a key enzyme of the oxidative pentose phosphate pathway (oxPPP), which forms a part of the central metabolism and plays an essential role in the response to environmental stress (Juhnke *et al.,* 1996; Averill *et al.,* 1998; Dennis and Blakeley, 2000). The 6-PGD expression is also increased in OFF leaves, as seen for G-3-PD. It has been found that 6-PGD activity also increased in *Dioscorea esculenta* tubers and *Curcuma longa* rhizomes prior to the visible appearance of sprouting and that it is most active during sprouting (Panneerselvam *et al.,* 2007). The last protein, namely fructosebisphosphate aldolase, catalyses the reaction of fructose-1,6-bisphosphate to dihydroxyacetone-phosphate and glyceraldehyde 3-phosphate. This enzyme is involved in the photoperiodic control of flowering in rice and its expression is also circadian-regulated in *Arabidopsis*. These data suggest that fructose-bisphosphate aldolase may prove to be a link between sugar metabolism and the flowering process (Matsumoto, 2006). Therefore, a direct relation between the up-expression of fructose-bisphosphate aldolase in the *Citrus* OFF buds and increased flowering in the following spring is feasible.

Proteins related to amino acid and protein metabolism

Other up-expressed proteins in the OFF-crop buds are those related to amino acid and protein metabolism, such as leucine-rich repeat transmembrane protein kinase (LRRP; spot 301); wheat adenosylhomocysteinase-like protein (spots 381 and 392); chaperonin (spots 274, 283 and 284); peptidyl-prolyl cis-trans isomerase (PPIase; spots 1079 and 1112). It is important to note that a member of the plant-specific LRRP (leucine-rich repeat transmembrane protein kinase) subfamily, *FLOR1* is up-expressed in the inflorescence meristem of *Arabidopsis* within 3 days of flowering induction.

FLOR1 seems to promote flowering by interacting with proteins, such as *SOC1*, which are expressed in the inflorescence meristem (Torti *et al.,* 2012). Thus, based on its up-regulation in the OFF-bud samples, the protein corresponding to spot 301 might act as *FLOR1*. Regarding the adenosylhomocysteinase-like protein, some plants are particularly sensitive to SAH-hydrolase (S-adenosylhomocysteinase-hydrolase) inhibition, which leads to the deregulation of flowering genes and alters flower morphology (Fulnecek *et al.,* 2011). Chaperonin is also of interest because Maeda *et al.* (2006) showed that in plumules of *Pharbitis nil* the up-regulation of a 60 kDa

chaperonin b-subunit may have a role in modulating other proteins during flower evocation; also, Zabaleta *et al.* (1994) observed that in tobacco plant with anti-sense Chaperonin 60 b, flowering was inhibited. Its expression is also higher in the 'Moncada' OFF-crop leaves (spot 358). Chaperonins are a type of molecular chaperones that occur in prokaryotes and in the mitochondria and plastids of eukaryotes (Hartl, 1996). Lastly, PPIase should also be noted because genes encoding a peptidy-prolyl cis/trans isomerase regulate the flowering time in *Arabidopsis* (Wang *et al.*, 2010).

Proteins related to oxidoreductase activity

Among these proteins up-expressed in OFF buds, multicopper oxidase (spot 251) and d-1-pyrroline-5-carboxylate reductase (spot 841) are highlighted. Regarding the former, the monocopper oxidase protein was also up-expressed in OFF leaves; copper oxidases are involved in plant growth processes, such as cell wall lignification or cotyledon vascular patterning (Jacobs and Roe, 2005). Lastly, d-1-pyrroline-5-carboxylate reductase seems to play a specific role in proline biosynthesis control (Nanjo *et al.*, 1999; Székely *et al.*, 2008); proline has been known to act as an osmoprotective molecule involved in maintaining the redox balance, ROS detoxification and the protection of protein structures (Hong *et al.*, 2000). The inhibition of proline biosynthesis by antisense d-1-pyrroline-5-carboxylate reductase causes severe abnormalities in flower development and vascular differentiation (Nanjo *et al.*, 1999; Székely *et al.*, 2008).

Other noteworthy up-expressed proteins in the off-crop samples

Up-expressed proteins found in OFF leaves and buds are adenylate isopentenyltransferase 8, chloroplastic (spot 1264 leaves), putative thiolase (spots 517 and 518 buds) in relation to the mevalonate pathway (Group [vii]), and both pectin methylesterase inhibitor PPE8B (spot 466 buds) and pectinesterase (spot 545 leaves and spot 625 buds), which are related to cell wall metabolism (Group [ix]). On the one hand, adenylate isopentenyltransferase catalyzes the initial step in the biosynthesis of cytokinin in higher plants (Takei *et al.*, 2001). Several recent studies highlight the importance of cytokinin content in primordial organs to control the progression of floral meristem development (Crane *et al.*, 2012; Kollmer *et al.*, 2011; Holst *et al.*, 2011). On

the other hand, the thiolase enzymes are involved in flowering because it is known that a mutant of 3-ketoacyl-CoA thiolase causes an alteration in the b-oxidation pathway in *Arabidopsis thaliana*, which prevents the initiation of floral meristems (Footitt *et al.*, 2007). Regarding the proteins related to cell wall metabolism, both pectin metylesterase (PME) and PME intracellular inhibitor, the up-expression in OFF samples can be explained by their implication in major physiological processes associated with reproductive plant development, including microsporogenesis and pollen tube growth (Bosch *et al.*, 2005; Jolie *et al.*, 2010).

Proteins up-expressed in the ON samples

Proteins related to primary metabolism

The largest group of proteins up-expressed in the ON buds are those related to carbohydrate metabolism and respiration. Among these, we stress the NADP-dependent malic enzyme (NADP-ME, spot 248) is interesting because it is also up-expressed in the ON-crop leaves (spots 515 and 516), and putative cytochrome c oxidase subunit II PS17 (COX II; spots 933, 967, 973, 998, 991, 1015, 1034, 1042, 1054, 1076, 1090, 1152, 1172 and 1214). The latter protein is the terminal enzyme of the mitochondrial electron transport chain, whose structure is still being defined (Millar *et al.*, 2004). The high level of COX II in the ON buds indicates that their respiratory capacity is greater as compared to the OFF buds (Lehtimäki *et al.*, 2011). Actively respiring plant mitochondria may produce ROS at high rates, although the accumulation of ROS and the induction of the ROS scavenging network enable the plant to survive upon toxin exposure; the up-regulation of the enzymatic defence system is likely to increase energy costs, reduced growth and ensure ultimate plant fitness.

Proteins with oxidoreductase activity

Contrary to what is observed in the ON samples, in the OFF samples, proteins with oxidoreductase activity are generally, down-regulated. Since in the spring following the collection of these samples, the flowering for OFF “Moncada” trees was significantly higher than for ON “Moncada” trees (Muñoz-Fambuena *et al.*, 2011), it is possible to establish a correlation between flower induction and a decline in

oxidoreductase activity in leaves. This possible correlation is based on the association observed in *Arabidopsis* between the developmental transition to flowering with a decline in the activity of its leaf ascorbate peroxidase (Banuelos *et al.,* 2008). Only the putative monocopper oxidase precursor was up-expressed in OFF samples. Copper oxidase genes belong to a large family and the main subgroups are ferroxidase, ascorbate oxidase and laccases. These proteins are involved in plant growth processes such as cell wall lignification or cotyledon vascular patterning (Jacobs and Roe, 2005).

Proteins related to stress/defense responses

Among these proteins, two should be highlighted: abscisic stress ripening-like protein (spot 812) and miraculin like protein (spots 837, 838, 850, 855, 890, 892, 913, 961, 981, 1151 and 1178). It should be noted that the abscisic stress ripening-like protein seems to play a role in flowering reversion (Chen *et al.,* 2009). Miraculin-like proteins are expressed abundantly in young mandarin fruits (Boo et al., 2007) and are likely to be involved in the defense response (this miraculin-like protein is also up-expressed in the ON leaves) (Tsukuda *et al.,* 2006).

Other noteworthy proteins up-expressed in the on-crop samples

Citrate synthase (spot 500) is particularly as it remarkable, is responsible for fruit acidity during its growth, increasing and then reaching a peak prior to ripening (Liu *et al.,* 2007; Sadka *et al.,* 2000). Another important protein up-expressed in the ON-crop sample is the cysteine proteinase-like protein (spot 1018), which plays a vital role in plant senescence and programming cell death; its inhibition is related to an increased number of flowers and seeds (Guerrero *et al.,* 1998; Solomon *et al.,* 1999). The down-expression of cysteine proteinase in OFF leaves samples may promote the flower development the following year.

In buds, chalcone-flavone isomerase (CFI), related to flavonoid biosynthesis (spot 882), is also remarkable. Some genes involved in flavonoid biosynthesis, including CFI, are rapidly down-regulated upon loss of paradormancy (Horvath *et al.,* 2005). This would explain why protein CFI is down-expressed in the OFF buds, which develop many more flowers than the ON buds in the following spring. Other authors have noted that six genes of the flavonoid metabolic pathway of the variety ‘Murcott’

(also very late mandarin), including chalcone isomerase, are induced in the OFF buds in May (Shalom *et al.*, 2012). It is possible that flavonoids in the bud serve as "sink" molecules for excess photoassimilates and for other carbon molecules that accumulate in trees in OFF years. Hence the present results seem to indicate that this behavior of flavonoids in the OFF buds reversed in November, at least in the 'Moncada' variety.

Proteins with unknown biological processes involved in (Group [x] in buds)

In this group, 9 up-expressed proteins in the OFF buds and 13 up-expressed proteins in the ON buds were identified. Some of these proteins have been previously isolated in GA-treated buds at the flowering induction time, such as spot 1041. None of these proteins was up-expressed in the 'Moncada' OFF or ON leaves.

In conclusion, many different proteins seem to be involved in alternate bearing. As far as this study shows, in the period of flowering induction, the primary metabolism is more active in OFF trees than in ON trees, depending on the proteins up-expressed in OFF leaves and buds. In contrast, in this same period the proteins up-expressed in ON samples compared to OFF samples are more closely related to the oxidoreductase activity and stress responses (either biotic and abiotic). However, these differences in the general metabolism of the plant may only be the consequence of the presence of fruits but not the cause of flowering inhibition. It seems logical that the tree accumulates starch reserves when the main sink (fruit) is not present in the tree (OFF-tree primary metabolism upregulation). On the other hand, the functional classification of stress responses is still imprecise. It is true that the fruit activates several proteins that are also activated during biotic or abiotic stress. However, to what extent can we consider the presence of fruits as a stress for the tree? The activation of these *stress-related* proteins (some of them related to ABA and JA) together with oxidoreductase activity (some of them related to ROS) might be the consequence of signalling mechanisms that the fruit imposes on the tree. Production of ROS, ABA ACC, and jasmonate are related to cell growth inhibition (Wolf *et al.*, 2012). This might be related to the main effect of the fruit in the tree as the most powerful sink, which is to regulate/inhibit vegetative growth (**Fig. 3.2**) (Martínez-Alcántara *et al.*, 2015). Further studies are needed to elucidate these relationships.

The activation of flowering gene inhibitors in ON-trees may be regulated by other mechanisms connected with these signalling processes. DNA methylation alters the interactions between proteins and DNA. Histone modification can alter the chromatin state by adding or removing methyl, acetyl, ubiquitin, or phosphate

modifications on histone tails (Strahl and Allis, 2000; Martinowich *et al.*, 2003). These epigenetic modifications are associated with gene expression regulation, providing plant cells with a mechanism to respond to developmental transitions.

Fruit influences DNA methylation and histone methyltranferases which trigger FLC gene expression in leaves during the flower induction period

The hypothesis studied is that the inhibition of flowering induction by the fruit is mediated by DNA methylation, as other authors have show that the promotion of flowering by prolonged exposure to low temperatures (vernalization) is mediated by DNA demethylation in *Arabidopsis thaliana* (Burn *et al.*, 1993; Finnegan *et al.*, 1998). The main conclusion found in this research is that methylation is observed in flowering inhibitors (to promote or silence its expression) but not in flowering promoters (*CiFT2*) which are regulated by flowering inhibitors and other endogenous mechanisms (i.e. hormones, see section 1).

During the flower induction period (November to January), the relative expression of *CiFT2* was higher in OFF leaves than in ON leaves (Muñoz-Fambuena *et al.*, 2011). On the contrary, all flowering inhibitors (*FLC*, *SVP* and *TEM1*; Lee *et al.*, 2007; Li *et al.*, 2008; Castillejo and Pelaz, 2008) showed higher relative expression in ON than in OFF leaves in September, before the induction period (November), and during the induction period (**Fig. 3.3**). In January, buds followed the same trend. However, two months later (May), after bud sprouting, the new shoots did not show significant differences in *CiFT2*, *FLC*, *SVP* and *TEM1* gene expressions. That is, buds and new leaves were restarted without any *inhibitory mark*. Consequently, DNA methylation profile was analyzed in the induction period (November) to explain differences between gene expression in ON and OFF leaves.

In plants, cytosine can be methylated at CG, CHG and CHH sites, where H represents any nucleotide but guanine (Capuano *et al.*, 2014). The promotor zone of each gene (*CiFT, FLC, SVP* and *TEM1*) was studied because the CG island (zone rich in CGs) in gene promoters acquires abnormal hypermethylation, which results in transcriptional silencing (Ehrlich *et al.*, 1982). This was mainly observed in the *TEM1* gene (**Fig. 3.5**). However, no differences were found in the methylated positions between ON and OFF leaves, which also showed the same gene expression in November (**Fig. 3.3**). *TEM* orthologs were isolated from antirrhinum (*AmTEM*) and

olive (*OeTEM*) and were expressed at their highest during their juvenile phase. These authors (Sgamma *et al.*, 2014) proposed that *TEM* might have a general role in regulating the juvenile vegetative phase in herbaceous and woody species.

In *Arabidopsis* the interaction between *SVP* and *FLC* mediated by various flowering genetic pathways governs the integration of flowering signals (Li *et al.*, 2008). Therefore, the promoter region of *SVP* was analyzed, and the result was that the presence of the fruit did not modify the DNA profile. *SVP* in the induction period showed demethylation in both ON and OFF leaves.

No methylation was found in the *CiFT1* gene in either ON or OFF leaves, whereas in *CiFT2* only one CG site showed partial methylation. In particular, in ON leaves, the *CiFT2* gene showed partial methylation (40%) in the first CG of the promoter region. This result seems not to be significant enough to determine the inhibition of the *CiFT2* expression in ON leaves, although DNA-methylation is a process of gene silencing (Matzke *et al.*, 2004). On the other hand, the *FLC* gene showed CG and CHH methylation in both ON and OFF leaves, indicating the existence of a fine-tuning mechanism that regulates protein accessibility to this gene (Zilberman *et al.*, 2006; Zhang *et al.*, 2006). In particular, *FLC* OFF leaves showed a CHH site partially methylated in the promoter zone, whereas ON leaves did not (**Fig. 3.5**). However, the main differences were found in the intron region: 7 CG sites were differentially methylated in ON and OFF leaves in November, when significant differences in *FLC* gene expression are observed (**Fig. 3.3**). On the other hand, in May, the same CG sites are equally methylated (or not) in ON and OFF leaves (except for the first CG site), and no differences are found in *FLC* gene expression. Therefore, it is thought that the fruit modifies DNA-methylation in *FLC* gene regulating its expression. Previously, other authors related changes in the methylation of *FLC,* either by vernalization (Finnegan *et al.*, 1998) or by the *MAF* gene family (Finnegan *et al.*, 2005), with the control of flowering in *Arabidopsis*.

The hypothesis for this research relating fruit induced DNA methylation and *FLC* expression is supported by the fact that DNA methyltransferase *ELF8* and histone methyltransferases *12ATX* and *7ATX* which activate *FLC* expression (He *et al.*, 2004; Tamada *et al.*, 2009) are significantly up-regulated in ON leaves in November. Promoters of the *FLC* expression by methylation activity, such as *ELF8,* are required to enhance histone 3 trimethylation at Lys 4 in *FLC* chromatin. This modification of *FLC* chromatin appears to be required to elevate *FLC* expression to levels that can

delay flowering in plants that have not been vernalized in *Arabidopsis* (He *et al.*, 2004); *ATX1* is involved in trimethylating histone H3-lysine 4 and it has been related with the activation of *FLC* (Pien *et al.*, 2008); *ATX2* is involved in dimethylating of histone H3-lysine 4. In the present study, only one candidate (*12ATX*) for these two genes was found, probably because the function is partially redundant (Saleh *et al.*, 2008). Finally, the gene *7ATX* is associated with increased *FLC* expression and H3K4 methylation in *Arabidopsis thaliana* (Tamada *et al.*, 2009). Based on the results described herein, only *ELF8* initiated its expression before *FLC*. *12ATX* and *7ATX* expression were also higher in ON than OFF leaves, but they were up-regulated after *FLC*. These results indicate that *ELF8* might activate the *FLC* expression and *12ATX* and *7ATX* might increase its expression.

On the other hand, inhibitors of *FLC* expression by methylation activity were also studied: *SKB1* and *5ATX* are required for epigenetic silencing of *FLC* (Wang *et al.*, 2007; Jacob *et al.*, 2009). In the experiments for this research *SKB1* relative expression was higher in OFF than ON trees in the induction period (November), this correlating with FLC inhibition.

Finally, this research's hypothesis is also supported by the defruiting experiment: 1) defruiting significantly reduced both *FLC* and methyltransferases, and promoted *CiFT2* and flowering; and 2) DNA methylation profile in defruited trees did not differ from the methylation profile in OFF trees.

Conclusions

In conclusion, flowering intensity in fruit trees is regulated by qualitative and quantitative coordinated mechanisms. In the ON-tree, hormones produced by the fruit (AUX, GA) act as signals in the leaves to demand a great amount of carbohydrates allowing for fruit development, and hampering vegetative growth. This signaling is constant throughout fruit development, and to a certain extent it might be *accumulative*. Thus, when the fruit reaches its final size, neighboring buds lose their ability to flower through an epigenetic mechanism (*switch-on, switch-off*): DNA-methylation of the *FLC* gene activates its expression, this reducing *CiFT2* gene expression and flowering regardless of exogenous promoting cues (i.e. low temperature). *CiFT2* gene expression is reduced but not completely switch-off (**Figure 6**).

In medium-yield trees, hormonal signaling does not arrive to many buds (branch independence). Therefore, some are able to flower although less than OFF-trees. In these medium-yield trees, flowering can be exogenously modified by hormonal treatments, either increasing flowering (with PBZ) or reducing it (with GA_3).

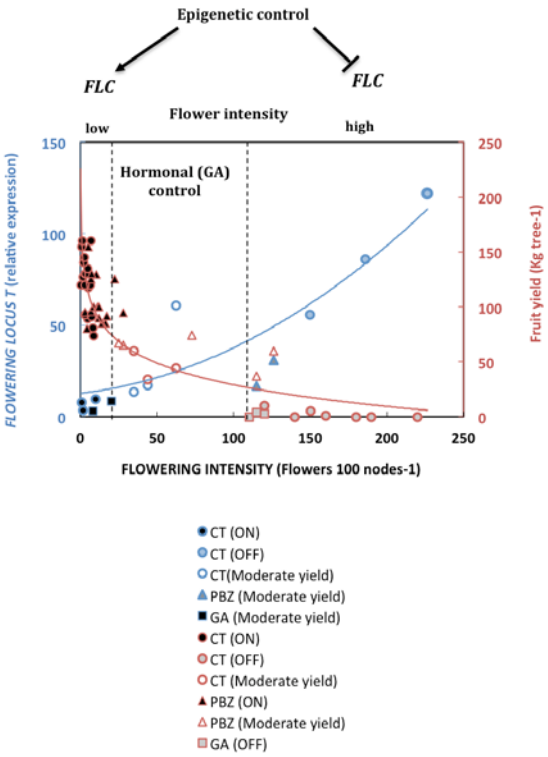


Figure 6. Flowering control in *Citrus* is regulated by fruit load, epigenetic control and hormonal (GA) control.

CT: control trees; PBZ: paclobutrazol-treated trees; GA: GA_3-treated trees; ON: heavy fruit-yield trees; OFF: low fruit-yield trees. *FLC*: *FLOWERING LOCUS C.*

Attending to the tested hypothesis and the objectives of this PhD Thesis, the main findings are:

1. The increase in *FLC* gene expression in ON-tree leaves coincides with fruit color change and low temperatures in the flower induction period. *CiFT2* is expressed in OFF-tree but not in ON-tree leaves.

2. The fruit produces and exports GAs and ABA to the leaves overlapping flower induction inhibition whereas these hormones decrease in OFF-tree leaves. GA_3 treatment reduces *CiFT2* gene expression but not *FLC* gene expression.

3. After bud sprouting, the inhibitory signals (*FLC*, *TEM1*, *SVP*) are not expressed in the new leaves. Every bud beside a fruit needs to restart vegetative growth to gain the flowering ability.

4. The fruit activates proteins from the stress responses and the oxidoreductase activity while in OFF-tree the primary metabolism and synthesis of starch is upregulated.

5. During the flower induction period, the fruit modifies the DNA-methylation profile of the *FLC* gene and increases the expression of methyltransferases that increase *FLC* gene expression. In defruited trees the process is reverted to the OFF-tree state.

References

Abassi, N. A., Kushad, M. M. and Endress, A. G. (1998). Active oxygen-scavenging enzymes activities in developing apple flowers and fruits. *Scientia Horticulturae*, *74*(3), 183-194.

Abbott, C. (1935). Blossom-bud differentiation in citrus trees. *American Journal of Botany*, 22, 476-485.

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H., Notaguchi, M., Goto, K. and Araki, T. (2005). *FD*, a bZIP protein mediating signals from the floral pathway integrator *FT* at the shoot apex. Science 309, 1052–1056.

Agustí, M. (1980). *Biología y control de la floración en el género Citrus* (Doctoral dissertation, Tesis Doctoral, Universitat Politècnica de València).

Agustí, M. (2003). *Citricultura* (No. 634.3 A398C 2003.). Madrid: Mundi-prensa.

Agustí, M., Almela, V. and Pons, J. (1992). Effects of girdling on alternate bearing in citrus. *J. Hort. Sci*, *67*(2), 203-210.

Albani, M. C. and Coupland, G. (2010). Chapter eleven-comparative analysis of flowering in annual and perennial plants. *Current topics in developmental biology*, *91*, 323-348.

Alquezar, B., Rodrigo, M. J. and Zacarías, L. (2008). Regulation of carotenoid biosynthesis during fruit maturation in the red-fleshed orange mutant Cara Cara. *Phytochemistry*, *69*(10), 1997-2007.

Alquézar, B., Zacarías, L. and Rodrigo, M. J. (2009). Molecular and functional characterization of a novel chromoplast-specific lycopene β-cyclase from Citrus and its relation to lycopene accumulation. *Journal of Experimental Botany*, *60*(6), 1783-1797.

Amasino, R. M. and Michaels, S. D. (2010). The timing of flowering. *Plant Physiology*, *154*(2), 516-520.

Andrés, F. and Coupland, G. (2012). The genetic basis of flowering responses to seasonal cues. *Nature Reviews Genetics*, *13*(9), 627-639.

Asin, L., Alegre, S. and Montserrat, R. (2007). Effect of paclobutrazol, prohexadione-Ca, deficit irrigation, summer pruning and root pruning on shoot growth, yield, and return bloom, in a 'Blanquilla'pear orchard. *Scientia horticulturae*, *113*(2), 142-148.

Averill, R. H., Bailey-Serres, J. and Kruger, N. J. (1998). Co-operation between cytosolic and plastidic oxidative pentose phosphate pathways revealed by 6-phosphogluconate dehydrogenase-deficient genotypes of maize. *The Plant Journal*, *14*(4), 449-457.

Ayalon, S. and Monselise, S. P. (1960). Flower bud induction and differentiation in the Shamouti orange. In *Proceedings. American Society for Horticultural Science* (Vol. 75, pp. 216-21).

Bangerth, F. (2000). Abscission and thinning of young fruit and thier regulation by plant hormones and bioregulators. *Plant Growth Regulation*, *31*(1-2), 43-59.

Bangerth, K. F. (2009). Floral induction in mature, perennial angiosperm fruit trees: similarities and discrepancies with annual/biennial plants and the involvement of plant hormones. *Scientia Horticulturae*, *122*(2), 153-163.

Baninasab, B. and Rahemi, M. (2006). Possible role of non-structural carbohydrates in alternate bearing of pistachio. *European journal of horticultural science*, 277-282.

Bañuelos, G. R., Argumedo, R., Patel, K., Ng, V., Zhou, F., and Vellanoweth, R. L. (2008). The developmental transition to flowering in Arabidopsis is associated with an increase in leaf chloroplastic lipoxygenase activity. *Plant science*, *174*(3), 366-373.

Bastow, R., Mylne, J. S., Lister, C., Lippman, Z., Martienssen, R. A. and Dean, C. (2004). Vernalization requires epigenetic silencing of *FLC* by histone methylation. *Nature*, *427*(6970), 164-167.

Battey, N. H. and Tooke, F. (2002). Molecular control and variation in the floral transition. *Current opinion in plant biology*, *5*(1), 62-68.

Bäurle, I. and Dean, C. (2006). The timing of developmental transitions in plants.*Cell*, *125*(4), 655-664.

Ben-Tal, Y. and Lavee, S. (1985). Girdling olive trees, a partial solution to biennial bearing. III. Chemical girdling: its influence on flowering and yield. *Rivista di ortoflorofrutticoltura italiana*, 1-11.

Bernier, G. (1971). Structural and metabolic changes in the shoot apex in transition to flowering. *Canadian Journal of Botany*, *49*(6), 803-819.

Bernier, G. and Pèrilleux, C. (2005). A physiological overview of the genetics of flowering time control. *Plant Biotechnology Journal*, *3*(1), 3-16.

Bertling, I. and Bangerth, F. (1995). Changes in hormonal pattern of the new growth of *Sclerocarya birrea* after rejuvenation treatment with GA_3 and" heading back". *Gartenbauwissenschaft*, *60*(3), 119-124.

Blaikie, S. J., Kulkarni, V. J. and Müller, W. J. (2004). Effects of morphactin and paclobutrazol flowering treatments on shoot and root phenology in mango cv. Kensington Pride. *Scientia Horticulturae*, *101*(1), 51-68.

Blázquez, M. A. and Weigel, D. (2000). Integration of floral inductive signals in *Arabidopsis*. *Nature*, *404*(6780), 889-892.

Blazquez, M. A., Ferrandiz, C., Madueno, F. and Parcy, F. (2006). How floral meristems are built. *Plant molecular biology*, *60*(6), 855-870.

Blázquez, M. A., Piñeiro, M. and Valverde, F. (2011). Bases moleculares de la floración. *Investigación y ciencia*.

Blondon, F. and Harada, H. (1972). Processus preparatoire et processus realisateur necessaires pour obtenir la floraison du Pharbitis nil Chois. *Acad Sci Paris CR Ser D*.

Blümel, M., Dally, N. and Jung, C. (2015). Flowering time regulation in crops—What did we learn from *Arabidopsis*?. *Current opinion in biotechnology*, *32*, 121-129.

Böhlenius, H., Huang, T., Charbonnel-Campaa, L., Brunner, A. M., Jansson, S., Strauss, S. H. and Nilsson, O. (2006). *CO/FT* regulatory module controls timing of flowering and seasonal growth cessation in trees. *Science*, *312*(5776), 1040-1043.

Boo, K. H., Kim, D. W., Cho, S. K., Jin, S. B., Kim, J. H., Lee, H. Y. and Riu, K. Z. (2007). Construction and profiling of a cDNA library from young fruit of satsuma mandarin. *Journal of Plant Biology*, *50*(4), 403-409.

Bosch, M., Cheung, A. Y., and Hepler, P. K. (2005). Pectin methylesterase, a regulator of pollen tube growth. *Plant Physiology*, *138*(3), 1334-1346.

Boss, P. K., Bastow, R. M., Mylne, J. S. and Dean, C. (2004). Multiple pathways in the decision to flower: enabling, promoting, and resetting. *The Plant Cell*, *16*(suppl 1), S18-S31.

Bünning, E. (1952). Über die ursachen der blühreife und blühperiodizität. *Z. Bot*,*40*, 293.

Burn, J. E., Bagnall, D. J., Metzger, J. D., Dennis, E. S., and Peacock, W. J. (1993). DNA methylation, vernalization, and the initiation of flowering.*Proceedings of the National Academy of Sciences*, *90*(1), 287-291.

Callejas, R. and Bangerth, F. (1997, April). Is auxin export of apple fruit an alternative signal for inhibition of flower bud induction?. In *VIII International Symposium on Plant Bioregulation in Fruit Production 463* (pp. 271-278).

Capuano, F., Mülleder, M., Kok, R., Blom, H. J. and Ralser, M. (2014). Cytosine DNA methylation is found in Drosophila melanogaster but absent in Saccharomyces cerevisiae, Schizosaccharomyces pombe, and other yeast species. *Analytical chemistry*, *86*(8), 3697-3702.

Carmona, M. J., Calonje, M. and Martínez-Zapater, J. M. (2007). The *FT/TFL1* gene family in grapevine. *Plant molecular biology*, *63*(5), 637-650.

Castillejo, C. and Pelaz, S. (2008). The balance between *CONSTANS* and *TEMPRANILLO* activities determines *FT* expression to trigger flowering.*Current Biology*, *18*(17), 1338-1343.

Centeno, D. C., Osorio, S., Nunes-Nesi, A., Bertolo, A. L., Carneiro, R. T., Araújo, W. L., Steinhauser, M. C., Michalska, J., Rohrmann, J., Geigenberger, P., Oliver. S. N., Stitt, M., Carrari, F., Rose, J. K. C. and Fernie, A. R. (2011). Malate plays a crucial role in starch metabolism, ripening, and soluble solid content of tomato fruit and affects postharvest softening. *The Plant Cell*, *23*(1), 162-184.

Chailakhyan, M. K. H. (1937). Hormonal theory of plant development. *Bull. Acad. Sci. URSS*.

Chan, B. G. and Cain, J. C. (1967, January). Effect of seed formation on subsequent flowering in apple. In *Proceedings of the American Society for Horticultural Science* (Vol. 91, No. DEC, p. 63).

Chen, S., Liu, H., Chen, W., Xie, D., and Zheng, S. (2009). Proteomic analysis of differentially expressed proteins in longan flowering reversion buds. *Scientia horticulturae*, *122*(2), 275-280.

Chica, E. J. and Albrigo, L. G. (2013). Expression of flower promoting genes in sweet orange during floral inductive water deficits. *Journal of the American Society for Horticultural Science*, *138*(2), 88-94.

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal*, *16*(6), 735-743.

Corbesier, L., Vincent, C., Jang, S. H., Fornara, F., Fan, Q. Z., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C. and Coupland, G. (2007). FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. *Science* 316, 1030–1033.

Costes, E., De Reffye, P., Lichou, J., Guédon, Y., Audubert, A. and Jay, M. (1992, February). Stochastic modelling of apricot growth units and branching. In *III International Symposium on Computer Modelling in Fruit Research and Orchard Management 313* (pp. 89-98).

Crane, O., Halaly, T., Pang, X., Lavee, S., Perl, A., Vankova, R. and Or, E. (2012). Cytokinin-induced VvTFL1A expression may be involved in the control of grapevine fruitfulness. *Planta*, *235*(1), 181-192.

Dag, A., Bustan, A., Avni, A., Tzipori, I., Lavee, S. and Riov, J. (2010). Timing of fruit removal affects concurrent vegetative growth and subsequent return bloom and yield in olive (*Olea europaea* L.). *Scientia horticulturae*, *123*(4), 469-472.

Davenport, T. L. (1983). Daminozide and gibberellin effects on floral induction of Citrus latifolia. *HortScience*, *18*(6), 947-949.

Davenport, T. L., Ying, Z., Kulkarni, V. and White, T. L. (2006). Evidence for a translocatable florigenic promoter in mango. *Scientia Horticulturae*, *110*(2), 150-159.

Davies, B. H. (1976). Carotenoids. In "Chemistry and biochemistry of plant pigments," ed. Goodwin, TW.

Davies, P. J. (2010). The plant hormones: their nature, occurrence, and functions. In *Plant hormones* (pp. 1-15). Springer Netherlands.

Deal, R. B., Topp, C. N., McKinney, E. C. and Meagher, R. B. (2007). Repression of flowering in *Arabidopsis* requires activation of *FLOWERING LOCUS C* expression by the histone variant H2A. Z. *The Plant Cell*, *19*(1), 74-83.

Delgado, R., Casamayor, R., Rodriguez, J. L., Cruz, P. and Fajardo, R. (1986). Paclobutrazol effects on oranges under tropical conditions. In *V International Symposium on Growth Regulators in Fruit Production 179* (pp. 537-544).

Dennis, D.T. and Blakeley, S.D. (2000). Carbohydrate metabolism, in: B.B. Buchanan, W. Gruissem, R.L. Jones (Eds.), *Biochemistry & Molecular Biology of Plants*, American Society of Plant Physiologists, Rockville, MD, pp. 652e654.

Dennis, F. G. and Neilsen, J. C. (1999). Physiological factors affecting biennial bearing in tree fruit: the role of seeds in apple. *HortTechnology*, *9*(3), 317-322.

Deronne, M. and Blondon, F. (1973). Mis en Cvidence de I'action inductrice de basses tem@ ratures, donnies continuellement en dysperiodes, sur la floraison du *Perilla ocyrnoides* L. Effect additif de cette action avec celle des jours courts. *CR Hebd. Seances Acad. Sci. Ser. D*, *276*, 3021-3024.

Dimmer, E. C., Huntley, R. P., Barrell, D. G., Binns, D., Draghici, S., Camon, E. B., Hubank M., Talmud, P. J., Apweiler, R. and Lovering, R. C. (2008). The gene ontology-providing a functional role in proteomic studies. *Proteomics*, *8*(23), NA.

Dornelas, M. C., Camargo, R. L. B., Figueiredo, L. H. M. and Takita, M. A. (2007). A genetic framework for flowering-time pathways in *Citrus* spp. *Genetics and Molecular Biology*, *30*(3), 769-779.

Ehrlich, M., Gama-Sosa, M. A., Huang, L. H., Midgett, R. M., Kuo, K. C., McCune, R. A. and Gehrke, C. (1982). Amount and distribution of 5-methylcytosine in human DNA from different types of tissues or cells. *Nucleic acids research*, *10*(8), 2709-2721.

El-Otmani, M., Goumari, M., Talhi, M., Wakkar, L., Srairi, I. and Lovatt, C. J. (2004). Effect of paclobutrazol on vegetative growth, flowering and fruiting of 'Nour'Clementine mandarin, an alternate bearing cultivar. In *Proc. Intl. Soc. Citriculture* (Vol. 2, pp. 543-545).

Endo, T., Shimada, T., Fujii, H., Kobayashi, Y., Araki, T. and Omura, M. (2005). Ectopic expression of an *FT* homolog from *Citrus* confers an early flowering phenotype on trifoliate orange (*Poncirus trifoliata* L. Raf.). *Transgenic research*, *14*(5), 703-712.

Endo, T., Shimada, T., Fujii, H., Nishikawa, F., Sugiyama, A., Nakano, M., Shimizu, T., Kobayashi, Y., Araki, T., Peña, L. and Omura, M. (2009). Development of a *CiFT* co-expression system for functional analysis of genes in citrus flowers and fruit. *Journal of the Japanese Society for Horticultural Science*, *78*(1), 74-83.

Erner, Y. (1988). Effects of girdling on the differentiation of inflorescence types and fruit set in 'Shamouti'orange trees. *Israel journal of botany*, *37*(2-4), 173-180.

Estornell, L. H., Agustí, J., Merelo, P., Talón, M. and Tadeo, F. R. (2013). Elucidating mechanisms underlying organ abscission. *Plant Science*, *199*, 48-60.

Fatta del Bosco, G. (1961). Indagini sull'epoca di differenziazione delle gemme nel nespolo del giappone. *Rivista di ortoflorofrutticoltura italiana*, 104-118.

Faure, S., Higgins, J., Turner, A. and Laurie, D. A. (2007). The *FLOWERING LOCUS T*-like gene family in barley (*Hordeum vulgare*). *Genetics*, *176*(1), 599-609.

Ferrándiz, C., Gu, Q., Martienssen, R. and Yanofsky, M. F. (2000). Redundant regulation of meristem identity and plant architecture by *FRUITFULL, APETALA1* and *CAULIFLOWER*. *Development*, *127*(4), 725-734.

Finnegan, E. J. and Dennis, E. S. (2007). Vernalization-induced trimethylation of histone H3 lysine 27 at *FLC* is not maintained in mitotically quiescent cells. *Current Biology*. 17, 1978–1983.

Finnegan, E. J., Genger, R. K., Kovac, K., Peacock, W. J. and Dennis, E. S. (1998). DNA methylation and the promotion of flowering by vernalisation. *Proceedings of the National Academy of Sciences USA* 95, 5824-5829

Finnegan, E. J., Kovac, K. A., Jaligot, E., Sheldon, C. C., Peacock, W. J. and Dennis, E. S. (2005). The downregulation of FLOWERING LOCUS C (FLC) expression in plants with low levels of DNA methylation and by vernalization occurs by distinct mechanisms. *The Plant Journal*, *44*(3), 420-432.

Footitt, S., Cornah, J. E., Pracharoenwattana, I., Bryce, J. H. and Smith, S. M. (2007). The Arabidopsis 3-ketoacyl-CoA thiolase-2 (kat2-1) mutant exhibits increased flowering but reduced reproductive success. *Journal of experimental botany*, *58*(11), 2959-2968.

Forshey, C. G. and Elfving, D. C. (1989). The relationship between vegetative growth and fruiting in apple trees. *Horticultural Reviews, Volume 11*, 229-287.

Foster, T., Johnston, R. and Seleznyova, A. (2003). A morphological and quantitative characterization of early floral development in apple (*Malus× domestica* Borkh.). *Annals of Botany*, *92*(2), 199-206.

Fulneček, J., Matyášek, R., Votruba, I., Holý, A., Křížová, K., and Kovařík, A. (2011). Inhibition of SAH-hydrolase activity during seed germination leads to deregulation of flowering genes and altered flower morphology in tobacco.*Molecular Genetics and Genomics*, *285*(3), 225-236.

Gambetta, G., Martínez-Fuentes, A., Bentancur, O., Mesejo, C., Reig, C., Gravina, A. and Agustí, M. (2012). Hormonal and nutritional changes in the flavedo regulating rind color development in sweet orange [*Citrus sinensis* (L.) Osb.]. *Journal of plant growth regulation*, *31*(3), 273-282.

Garcia-Luis, A., Almela, V., Monerri, C., Agustí, M. and Guardiola, J. L. (1986). Inhibition of flowering in vivo by existing fruits and applied growth regulators in *Citrus unshiu*. *Physiologia plantarum*, *66*(3), 515-520.

Giakountis, A. and Coupland, G. (2008). Phloem transport of flowering signals. *Current opinion in plant biology*, *11*(6), 687-694.

Gifford, E. M. and Corson, G. E. (1971). The shoot apex in seed plants. *The Botanical Review*, *37*(2), 143-229.

Goldberg-Moeller, R., Shalom, L., Shlizerman, L., Samuels, S., Zur, N., Ophir, R., Blumwald, E. and Sadka, A. (2013). Effects of gibberellin treatment during flowering induction period on global gene expression and the transcription of flowering-control genes in *Citrus* buds. *Plant science*, *198*, 46-57.

Goldschmidt, E. E. and Golomb, A. (1982). The carbohydrate balance of alternate-bearing citrus trees and the significance of reserves for flowering and fruiting. *Journal of the American Society of Horticultural Science*, *107*(2), 206-208.

Goldschmidt, E. E. and Monselise, S. P. (1970). Hormonal control of flowering in citrus and some other woody perennials. In *Plant growth substances.* Springer Berlin Heidelberg, pp. 758-766.

Goldschmidt, E. E. and Samach, A. (2001, August). Aspects of flowering in fruit trees. In *IX International Symposium on Plant Bioregulators in Fruit Production 653* (pp. 23-27).

Gómez-Cadenas, A., Mehouachi, J., Tadeo, F. R., Primo-Millo, E. and Talon, M. (2000). Hormonal regulation of fruitlet abscission induced by carbohydrate shortage in citrus. *Planta*, *210*(4), 636-643.

Gorsuch, P. A., Pandey, S. and Atkin, O. K. (2010). Temporal heterogeneity of cold acclimation phenotypes in *Arabidopsis* leaves. *Plant, cell & environment*, *33*(2), 244-258.

Griggs, W. H., Martin, G. C. and Iwakiri, B. T. (1970). The effect of seedless versus seeded fruit development on flower bud formation in pear. *Journal of the American Society of Horticultural Science*, *95*, 243-8.

Grodzinski, B., Jiao, J., Knowles, V. L. and Plaxton, W. C. (1999). Photosynthesis and carbon partitioning in transgenic tobacco plants deficient in leaf cytosolic pyruvate kinase. *Plant physiology*, *120*(3), 887-896.

Guardiola, J. L., Monerri, C. and Agusti, M. (1982). The inhibitory effect of gibberellic acid on flowering in *Citrus*. *Physiol. Plant*, *55*(2), 136-142.

Guerrero, C., de la Calle, M., Reid, M. S. and Valpuesta, V. (1998). Analysis of the expression of two thiolprotease genes from daylily (Hemerocallis spp.) during flower senescence. *Plant molecular biology*, *36*(4), 565-571.

Gyllenstrand, N., Clapham, D., Källman, T. and Lagercrantz, U. (2007). A Norway spruce *FLOWERING LOCUS T* homolog is implicated in control of growth rhythm in conifers. *Plant physiology*, *144*(1), 248-257.

Hackett, W. P. (1985). Juvenility, maturation, and rejuvenation in woody plants.*Horticultural Reviews, Volume 7*, 109-155.

Handschack, M. and Schmidt, S. (1990). Alternate bearing in apple as influenced by the climate in Central Europe, 1: Maximum and minimum temperatures. *Archiv fuer Gartenbau (German DR)*.

Hartl, F. U. (1996). Molecular chaperones in cellular protein folding.

Harty, A. R. and Van Staden, J. (1988). Paclobutrazol and temperature effects on lemon. In *Citriculture: proceedings of the Sixth International Citrus Congress: Middle-East, Tel Aviv, Israel, March 6-11, 1988/scientific editors, R. Goren and K. Mendel, editor, N. Goren*. Rehovot, Israel: Balaban, c1989.

Hayama, R., Agashe, B., Luley, E., King, R. and Coupland, G. (2007). A circadian rhythm set by dusk determines the expression of *FT* homologs and the short-day photoperiodic flowering response in *Pharbitis*. *The Plant Cell*, *19*(10), 2988-3000.

He, Y., Doyle, M. R. and Amasino, R. M. (2004). PAF1-complex-mediated histone methylation of *FLOWERING LOCUS C* chromatin is required for the vernalization-responsive, winter-annual habit in Arabidopsis. *Genes & development*, *18*(22), 2774-2784.

Hedden, P., Hoad, G. V., Gaskin, P., Lewis, M. J., Green, J. R., Furber, M. and Mander, L. N. (1993). Kaurenoids and gibberellins, including the newly characterized gibberellin A 88, in developing apple seeds. *Phytochemistry*, *32*(2), 231-237.

Hepworth, S. R., Valverde, F., Ravenscroft, D., Mouradov, A. and Coupland, G. (2002). Antagonistic regulation of flowering-time gene SOC1 by *CONSTANS* and *FLC* via separate promoter motifs. *The EMBO Journal*, *21*(16), 4327-4337.

Hoad, G. V., Retamales, J. A., Whiteside, R. J. and Lewis, M. J. (1993). Phloem translocation of gibberellins in three species of higher plants. *Plant growth regulation*, *13*(1), 85-88.

Hoagland, D. R. (1920). Optimum nutrient solutions for plants. *Science*, 562-564.

Holst, K., Schmülling, T. and Werner, T. (2011). Enhanced cytokinin degradation in leaf primordia of transgenic Arabidopsis plants reduces leaf size and shoot organ primordia formation. *Journal of plant physiology*, *168*(12), 1328-1334.

Hong, Z., Lakkineni, K., Zhang, Z., and Verma, D. P. S. (2000). Removal of feedback inhibition of Δ1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress.*Plant Physiology*, *122*(4), 1129-1136.

Horvath, D. P., Soto-Suárez, M., Chao, W. S., Jia, Y. and Anderson, J. V. (2005). Transcriptome analysis of paradormancy release in root buds of leafy spurge (Euphorbia esula). *Weed science*, *53*(6), 795-801.

Hsu, C. Y., Adams, J. P., Kim, H., No, K., Ma, C., Strauss, S. H., Dmevich, J., Vandervelde, L., Ellis, J. D., Rice, B., Wickett, N., Gunter, L. E., Tuskan, G. A., Brunner, A. M., Page, G. P., Barakat, A., Carlson, J. E., dePamphilis, C. W., Luthe, D. S. and Yuceer, C. (2011). *FLOWERING LOCUS T* duplication coordinates reproductive and vegetative growth in perennial poplar. *Proceedings of the National Academy of Sciences*, *108*(26), 10756-10761.

Huet, J. (1972, August). Floral initiation in pear trees. In *Symposium on growth Regulators in Fruit Production 34* (pp. 193-198).

Inoue, H. (1990). Effects of temperature on bud dormancy and flower bud differentiation in satsuma mandarin. *Journal of the Japanese Society for Horticultural Science*, *58*(4), 919-926.

Ito, A., Hayama, H. and Yoshioka, H. (2001). The effect of shoot-bending on the amount of diffusible indole-3-acetic acid and its transport in shoots of Japanese pear. *Plant growth regulation*, *34*(2), 151-158.

Iwasaki, T. (1959). Studies on the differentiation and development of the flower bud in citrus. *Bulletin of Tokai-Kinki Agricultural Experiment Station of Horticulture*, *5*, 4-35.

Jack, T. (2004). Molecular and genetic mechanisms of floral control. *The Plant Cell*, *16*(suppl 1), S1-S17.

Jacob, Y., Feng, S., LeBlanc, C. A., Bernatavichute, Y. V., Stroud, H., Cokus, S., Johnson, L. M., Pellegrini, M., Jacobsen, S. E. and Michaels, S. D. (2009). *ATXR5* and *ATXR6* are H3K27 monomethyltransferases required for chromatin structure and gene silencing.*Nature structural & molecular biology*, *16*(7), 763-768.

Jacobs, J., and Roe, J. L. (2005). SKS6, a multicopper oxidase-like gene, participates in cotyledon vascular patterning during Arabidopsis thaliana development. *Planta*, *222*(4), 652-666.

Jacqmard, A., Miksche, J. P. and Bernier, G. (1972). Quantitative study of nucleic acids and proteins in the shoot apex of *Sinapis alba* during transition from the vegetative to the reproductive condition. *American Journal of Botany*, 714-721.

Jang, S., Torti, S. and Coupland, G. (2009). Genetic and spatial interactions between *FT, TSF* and *SVP* during the early stages of floral induction in *Arabidopsis*. *Plant Journal*. 60, 614–625.

Jolie, R. P., Duvetter, T., Van Loey, A. M., and Hendrickx, M. E. (2010). Pectin methylesterase and its proteinaceous inhibitor: a review. *Carbohydrate Research*, *345*(18), 2583-2595.

Juhnke, H., Krems, B., Kötter, P. and Entian, K. D. (1996). Mutants that show increased sensitivity to hydrogen peroxide reveal an important role for the pentose phosphate pathway in protection of yeast against oxidative stress. *Molecular and General Genetics MGG*, *252*(4), 456-464.

Julien, M. H. R. (1973). Physiology of flowering in *Saccharum I.* Daylength control of floral initiation and development in *S. spontaneum* L. *Journal of Experimental Botany*, *24*(3), 549-557.

Kardailsky, I., Shukla, V. K., Ahn, J. H., Dagenais, N., Christensen, S. K., Nguyen, J. T., Chory, J., Harrison, M. J. and Weigel, D. (1999). Activation tagging of the floral inducer *FT*. *Science* 286, 1962–1965.

Katayama, T. C. (1971). Photoperiodism in the Genus *Oryza*. III. *Memoirs of the Faculty of Agriculture, Kagoshima University*, *8*(1), 299-320.

Kawasaki, T., Mizuno, K., Shimada, H., Satoh, H., Kishimoto, N., Okumura, S., Ichikawa, N. and Baba, T. (1996). Coordinated regulation of the genes participating in starch biosynthesis by the rice floury-2 locus. *Plant physiology*, *110*(1), 89-96.

Kinet, J. M. (1993). Environmental, chemical, and genetic control of flowering. *Horticultural Reviews*, *15*, 279-334.

King, R. W. and Evans, L. T. (2005). Florigen or florigenie(s). *Flowering Newslett*. 40, 27–30.

King, R. W. and Zeevaart, J. A. (1973). Floral stimulus movement in *Perilla* and flower inhibition caused by noninduced leaves. *Plant Physiology*, *51*(4), 727-738.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. and Araki, T. (1999). A pair of related genes with antagonistic roles in mediating flowering signals. *Science*, *286*(5446), 1960-1962.

Kobel, F. (1954). *Lehrbuch des Obstbaus auf physiologischer Grundlage*. Springer-Verlag.

Köllmer, I., Werner, T., and Schmülling, T. (2011). Ectopic expression of different cytokinin-regulated transcription factor genes of Arabidopsis thaliana alters plant growth and development. *Journal of plant physiology*, *168*(12), 1320-1327.

Komiya, R., Ikegami, A., Tamaki, S., Yokoi, S. and Shimamoto, K. (2008). *Hd3a* and *RFT1* are essential for flowering in rice. *Development*, *135*(4), 767-774.

Koshita, Y. and Takahara, T. (2004). Effect of water stress on flower-bud formation and plant hormone content of satsuma mandarin (*Citrus unshiu* Marc.). *Scientia horticulturae*, *99*(3), 301-307.

Koshita, Y., Takahara, T., Ogata, T. and Goto, A. (1999). Involvement of endogenous plant hormones (IAA, ABA, GAs) in leaves and flower bud formation of satsuma mandarin (*Citrus unshiu* Marc.). *Scientia Horticulturae*, *79*(3), 185-194.

Kotoda, N., Hayashi, H., Suzuki, M., Igarashi, M., Hatsuyama, Y., Kidou, S. I., Igasaki, T., Nishiguchi, M., Yano, K., Shimizu, T., Takahashi, S., Iwanami, H., Moriya, S. and Abe, K. (2010). Molecular characterization of *FLOWERING LOCUS T*-like genes of apple (*Malus× domestica* Borkh.). *Plant and Cell Physiology*, *51*(4), 561-575.

Kotoda, N., Wada, M., Komori, S., Kidou, S. I., Abe, K., Masuda, T. and Soejima, J. (2000). Expression pattern of homologues of floral meristem identity genes *LFY* and *AP1* during flower development in apple. *Journal of the American Society for Horticultural Science*, *125*(4), 398-403.

Knowles, V. L., McHugh, S. G., Hu, Z., Dennis, D. T., Miki, B. L. and Plaxton, W. C. (1998). Altered growth of transgenic tobacco lacking leaf cytosolic pyruvate kinase. *Plant physiology*, *116*(1), 45-51.

Krajewski, A. J. and Rabe, E. (1995). Citrus flowering: a critical evaluation. *Journal of Horticultural Science (United Kingdom)*.

Lang, A. (1965). *Physiology of flower initiation* (pp. 1380-1536). Springer Berlin Heidelberg.

Lavee, S. (1988, July). Involvement of plant growth regulators and endogenous growth substances in the control of alternate bearing. In *VI International Symposium on Growth Regulators in Fruit Production 239* (pp. 311-322).

Lee, H., Suh, S. S., Park, E., Cho, E., Ahn, J. H., Kim, S.G., Lee, J.S., Kwon, Y.M. and Lee, I. (2000). The *AGAMOUS*-LIKE 20 MADS domain protein integrates floral inductive pathways in *Arabidopsis. Genes and Development* 14, 2366-2376

Lee, J. H., Yoo, S. J., Park, S. H., Hwang, I., Lee, J. S. and Ahn, J. H. (2007). Role of *SVP* in the control of flowering time by ambient temperature in *Arabidopsis*. *Genes and development*, *21*(4), 397-402.

Lee, J., Oh, M., Park, H. and Lee, I. (2008). *SOC1* translocated to the nucleus by interaction with *AGL24* directly regulates *LEAFY*. *The Plant Journal*, *55*(5), 832-843.

Leegood, R. C. (1996). Primary photosynthate production: physiology and metabolism. *Photoassimilate distribution in plants and crops. Source-sink relationships.* Zamski, E. & Schaffer, *AA (Ed.)*, Marcel Dekker, New York, 21-42.

Lehtimäki, N., Shunmugam, S., Jokela, J., Wahlsten, M., Carmel, D., Keränen, M., Sivonen, K., Aro, E. M., Allahverdiyeva, Y. and Mulo, P. (2011). Nodularin uptake and induction of oxidative stress in spinach (Spinachia oleracea). *Journal of plant physiology*, *168*(6), 594-600.

Li, D., Liu, C., Shen, L., Wu, Y., Chen, H., Robertson, M., Helliwell, C. A., Ito, T., Meyerowitz, E. and Yu, H. (2008). A repressor complex governs the integration of flowering signals in Arabidopsis. *Developmental cell*, *15*(1), 110-120.

Li, S. H., Meng, Z. Q., Li, T. H., Liu, H. Z. and Tu, Y. C. (1995). Critical period of flower bud induction in Red Fuji and Ralls Janet apple trees.*Gartenbauwissenschaft (Germany)*.

Lifschitz, E., Eviatar, T., Rozman, A., Shalit, A., Goldshmidt, A., Amsellem, Z., Alvarez, J. P. and Eshed, Y. (2006). The tomato *FT* ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. *Proceedings of the National Academy of Sciences*, *103*(16), 6398-6403.

Lin, M. K., Belanger, H., Lee, Y. J., Varkonyi-Gasic, E., Taoka, K. I., Miura, E., Xoconostle-Cazares, B., Gendler, K., Jorgensene, R. A., Phinney, B., Lough, T. J. and Lucas, W. J. (2007). *FLOWERING LOCUS T* protein may act as the long-distance florigenic signal in the cucurbits. *Plant Cell* 19, 1488–1506.

Liu, C., Xi, W. Y., Shen, L. S., Tan, C. P. and Yu, H. (2009). Regulation of floral patterning by flowering time genes. *Dev. Cell* 16, 711–722.

Liu, Y., Liu, Q., Xiong, J. and Deng, X. (2007). Difference of a citrus late-ripening mutant (Citrus sinensis) from its parental line in sugar and acid metabolism at the fruit ripening stage. *Science in China Series C: Life Sciences*, *50*(4), 511-517.

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods*, *25*(4), 402-408.

Lombard, P. J., Cook, N. C. and Bellstedt, D. U. (2006). Endogenous cytokinin levels of table grape vines during spring budburst as influenced by hydrogen cyanamide application and pruning. *Scientia horticulturae*, *109*(1), 92-96.

Longman, K. A., Nasr, T. A. A. and Wareing, P. F. (1965). Gravimorphism in trees 4. The effect of gravity on flowering. *Annals of Botany*, *29*(3), 459-473.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J Biol Chem*, *193*(1), 265-275.

Luckwill, L. C. (1969). Control of growth and fruitfulness of apple trees. In *Long Ashton Symp, 2d, Univ of Bristol*.

Maeda, T., Mori, H., Shiratake, K., Yamada, K., Takeno, K., and Yamaki, S. (2006). cDNA macroarray analysis of genes expressed in plumules of Pharbitis nil after induction of flowering. *Journal of horticultural science & biotechnology*,*81*(3), 496-500.

Mandel, M. A. and Yanofsky, M. F. (1995). A gene triggering flower formation in *Arabidopsis*. *Nature*, *377*(6549), 522-524.

Martínez-Alcántara, B., Iglesias, D. J., Reig, C., Mesejo, C., Agustí, M. and Primo-Millo, E. (2015). Carbon utilization by fruit limits shoot growth in alternate-bearing citrus trees. *Journal of plant physiology*, *176*, 108-117.

Martínez-Fuentes, A., Mesejo, C., Juan, M., Almela, V. and Agustí, M. (2004). Restrictions on the exogenous control of flowering in citrus. In *XXVI International Horticultural Congress: Citrus and Other Subtropical and Tropical Fruit Crops: Issues, Advances and 632* pp. 91-98.

Martínez-Fuentes, A., Mesejo, C., Reig, C. and Agustí, M. (2010). Timing of the inhibitory effect of fruit on return bloom of 'Valencia'sweet orange (*Citrus sinensis* (L.) Osbeck). *Journal of the Science of Food and Agriculture*, *90*(11), 1936-1943.

Martínez-Zapater, J.M., Coupland, G., Dean, C. and Koornneef, M. (1994). The transition of flowering in *Arabidopsis*. In: Meyerowitz EM, Somerville CR (Eds) *Arabidopsis*, Cold Spring Harbour Laboratory Press, New York, pp 430-434.

Martinowich, K., Hattori, D., Wu, H., Fouse, S., He, F., Hu, Y., Fan G. and Sun, Y. E. (2003). DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science*, *302*(5646), 890-893.

Martín-Trillo, M. and Martínez-Zapater, J. M. (2002). Growing up fast: manipulating the generation time of trees. *Current opinion in biotechnology*, *13*(2), 151-155.

Maserti, B. E., Del Carratore, R., Della Croce, C. M., Podda, A., Migheli, Q., Froelicher, Y., Luro, F., Morillon, R.., Ollitrault, P., Talon, M. and Rossignol, M. (2011). Comparative analysis of proteome changes induced by the two spotted spider mite *Tetranychus urticae* and methyl jasmonate in citrus leaves. *Journal of plant physiology*, *168*(4), 392-402.

Mathieu, J., Warthmann, N., Küttner, F. and Schmid, M. (2007). Export of FT protein from phloem companion cells is sufficient for floral induction in *Arabidopsis*. *Current Biology*, *17*(12), 1055-1060.

Matsumoto, T. K. (2006). Genes uniquely expressed in vegetative and potassium chlorate induced floral buds of Dimocarpus longan. *Plant science*,*170*(3), 500-510.

Matzke, M., Aufsatz, W., Kanno, T., Daxinger, L., Papp, I., Mette, M. F. and Matzke, A. J. (2004). Genetic analysis of RNA-mediated transcriptional gene silencing. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, *1677*(1), 129-141.

Michaels, S. D., Himelblau, E., Kim, S. Y., Schomburg, F. M. and Amasino, R. M. (2005). Integration of flowering signals in winter-annual Arabidopsis. *Plant Physiology*, *137*(1), 149-156.

Millar, A. H., Eubel, H., Jänsch, L., Kruft, V., Heazlewood, J. L. and Braun, H. P. (2004). Mitochondrial cytochrome c oxidase and succinate dehydrogenase complexes contain plant specific subunits. *Plant molecular biology*, *56*(1), 77-90.

Monerri, C., Fortunato-Almeida, A., Molina, R. V., Nebauer, S. G., Garcia-Luis, A. and Guardiola, J. L. (2011). Relation of carbohydrate reserves with the forthcoming crop, flower formation and photosynthetic rate, in the alternate bearing 'Salustiana'sweet orange (*Citrus sinensis* L.). *Scientia Horticulturae*, *129*(1), 71-78.

Monselise, S. P. and Goldschmidt, E. E. (1982). Alternate Bearing in Fruit Trees 1. *Horticultural reviews*, *4*, 128-173.

Monselise, S. P. and Halevy, A. H. (1964). Chemical Inhibition and Promotion of *Citrus* Flower Bud Induction1, 2.

Monteireo da Cruz, M. D., Siqueira, D. L. D., Salomão, L. C. C. and Cecon, P. R. (2008). Influência do paclobutrazol e da temperatura ambiente sobre o florescimento e frutificação da limeira ácida 'Tahiti'. *Ciência Agrotec*, *32*(4), 1148-1153.

Moon, J., Suh, S. S., Lee, H., Choi, K. R., Hong, C. B., Paek, N. C., Kim, S. and Lee, I. (2003). The *SOC1* MADS-box gene integrates vernalization and gibberellin signals for flowering in *Arabidopsis. The Plant Journal*, *35*(5), 613-623.

Moss, G. I. (1970). Chemical control of flower development in sweet orange (*Citrus sinensis*). *Crop and Pasture Science*, *21*(2), 233-242.

Muday, G. K. and DeLong, A. (2001). Polar auxin transport: controlling where and how much. *Trends in plant science*, *6*(11), 535-542.

Muñoz-Fambuena, N., Mesejo, C., González-Mas, M. C., Iglesias, D. J., Primo-Millo, E. and Agustí, M. (2012a). Gibberellic acid reduces flowering intensity in sweet orange [*Citrus sinensis* (L.) Osbeck] by repressing *CiFT* gene expression. *Journal of Plant Growth Regulation*, *31*(4), 529-536.

Muñoz-Fambuena, N., Mesejo, C., González-Mas, M. C., Primo-Millo, E., Agustí, M. and Iglesias, D. J. (2011). Fruit regulates seasonal expression of flowering genes in alternate-bearing 'Moncada'mandarin. *Annals of Botany*, *108*(3), 511-519.

Muñoz-Fambuena, N., Mesejo, C., González-Mas, M. C., Primo-Millo, E., Agustí, M. and Iglesias, D. J. (2012b). Fruit load modulates flowering-related gene expression in buds of alternate-bearing 'Moncada'mandarin. *Annals of botany*,*110* (6), 1109-1118.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum*, *15*(3), 473-497.

Mutasa-Göttgens, E. and Hedden, P. (2009). Gibberellin as a factor in floral regulatory networks. *Journal of Experimental Botany*, erp 040.

Mylne, J. S., Barrett, L., Tessadori, F., Mesnage, S., Johnson, L., Bernatavichute, Y. V., Jacobsen, S. E., Fransz, P. and Dean, C. (2006). *LHP1*, the *Arabidopsis* homologue of *HETEROCHROMATIN PROTEIN1*, is required for epigenetic silencing of *FLC*. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(13), 5012-5017.

Nakagawa, M., Honsho, C., Kanzaki, S., Shimizu, K. and Utsunomiya, N. (2012). Isolation and expression analysis of *FLOWERING LOCUS T*-like and gibberellin metabolism genes in biennial-bearing mango trees. *Scientia horticulturae*, *139*, 108-117.

Nanjo, T., Kobayashi, M., Yoshiba, Y., Sanada, Y., Wada, K., Tsukaya, H., Kakubari, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1999). Biological functions of proline in morphogenesis and osmotolerance revealed in antisense transgenic Arabidopsis thaliana. *The Plant Journal*, *18*(2), 185-193.

Nelissen, H., Boccardi, T. M., Himanen, K. and Van Lijsebettens, M. (2007). Impact of core histone modifications on transcriptional regulation and plant growth. *Critical reviews in plant sciences*, *26*(5-6), 243-263.

Nelissen, H., Rymen, B., Jikumaru, Y., Demuynck, K., Van Lijsebettens, M., Kamiya, Y., Inze, D. and Beemster, G. T. (2012). A local maximum in gibberellin levels regulates maize leaf growth by spatial control of cell division. *Current Biology*, *22*(13), 1183-1187.

Nieminen, K., Immanen, J., Laxell, M., Kauppinen, L., Tarkowski, P., Dolezal, K., Tähtiharju, S., Elo, A., Decourteix, M., Ljung, K., Bhalerao, R., Keinonen, K., Albert, V. A. and Helariutta, Y. (2008). Cytokinin signaling regulates cambial development in poplar. *Proceedings of the National Academy of Sciences*, *105*(50), 20032-20037.

Nishikawa, F. (2013). Regulation of Floral Induction in *Citrus*. *Journal of the Japanese Society for Horticultural Science*, *82*(4), 283-292.

Nishikawa, F., Endo, T., Shimada, T., Fujii, H., Shimizu, T. and Omura, M. (2009). Differences in seasonal expression of flowering genes between deciduous trifoliate orange and evergreen Satsuma mandarin. *Tree physiology*, *29*(7), 921-926.

Nishikawa, F., Endo, T., Shimada, T., Fujii, H., Shimizu, T., Omura, M. and Ikoma, Y. (2007). Increased *CiFT* abundance in the stem correlates with floral induction by low temperature in Satsuma mandarin (*Citrus unshiu* Marc.). *Journal of experimental botany*, *58*(14), 3915-3927.

Nishikawa, F., Iwasaki, M., Fukamachi, H. and Endo, T. (2013). Leaf removal suppresses citrus *FLOWERING LOCUS T* expression in satsuma mandarin. *Bull. Natl. Fruit Tree Sci*, *15*, 1-6.

Nishikawa, F., Iwasaki, M., Fukamachi, H., Nonaka, K., Imai, A. and Endo, T. (2011). Seasonal changes of citrus *Flowering Locus T* gene expression in kumquat. *Bull Natl Inst Fruit Tree Sci*, *12*, 27-32.

Nishikawa, F., Iwasaki, M., Fukamachi, H., Nonaka, K., Imai, A., Takishita, F., Yano, T. and Endo, T. (2012). Fruit Bearing Suppresses *Citrus FLOWERING LOCUS T* Expression in Vegetative Shoots of Satsuma Mandarin (*Citrus unshiu* Marc.). *Journal of the Japanese Society for Horticultural Science*, *81*(1), 48-53.

Ogata, T., Hasukawa, H., Shiozaki, S., Horiuchi, S., Kawase, K., Iwagaki, I. and Okuda, H. (1996). Seasonal changes in endogenous gibberellin contents in satsuma mandarin [Citrus reticulata] during flower differentiation and the influence of paclobutrazol on gibberellin synthesis. *Journal of the Japanese Society for Horticultural Science (Japan)*.

Oliveira, C. M. and Browning, G. (1993). Gibberellin structure-activity effects on flower initiation in mature trees and on shoot growth in mature and juvenile *Prunus avium*. *Plant growth regulation*, *13*(1), 55-63.

Oparka, K. J. and Cruz, S. S. (2000). The great escape: phloem transport and unloading of macromolecules 1. *Annual review of plant biology*, *51*(1), 323-347.

Panneerselvam, R., Jaleel, C. A., Somasundaram, R., Sridharan, R. and Gomathinayagam, M. (2007). Carbohydrate metabolism in Dioscorea esculenta (Lour.) Burk. tubers and Curcuma longa L. rhizomes during two phases of dormancy. *Colloids and Surfaces B: Biointerfaces*, *59*(1), 59-66.

Parcy, F. (2005). Flowering: a time for integration. *International Journal of Developmental Biology*, *49*(5/6), 585.

Peña, L., Martín-Trillo, M., Juárez, J., Pina, J. A., Navarro, L. and Martínez-Zapater, J. M. (2001). Constitutive expression of *Arabidopsis LEAFY* or *APETALA1* genes in citrus reduces their generation time. *Nature biotechnology*, *19*(3), 263-267.

Pharis, R. P. and King, R. W. (1985). Gibberellins and reproductive development in seed plants. *Annual Review of Plant Physiology*, *36*(1), 517-568.

Pien, S., Fleury, D., Mylne, J. S., Crevillen, P., Inzé, D., Avramova, Z., Dean, C. and Grossniklaus, U. (2008). *ARABIDOPSIS TRITHORAX1* dynamically regulates *FLOWERING LOCUS C* activation via histone 3 lysine 4 trimethylation. *The Plant Cell*, *20*(3), 580-588.

Pillitteri, L. J., Lovatt, C. J. and Walling, L. L. (2004a). Isolation and characterization of a *TERMINAL FLOWER* homolog and its correlation with juvenility in citrus. *Plant Physiology*, *135*(3), 1540-1551.

Pillitteri, L. J., Lovatt, C. J. and Walling, L. L. (2004b). Isolation and Characterization of *LEAFY* and *APETALA1* Homologues from *Citrus sinensis* L. Osbeck Washington'. *JOURNAL-AMERICAN SOCIETY FOR HORTICULTURAL SCIENCE*, *129*, 846-856.

Pin, P. A. and Nilsson, O. (2012). The multifaceted roles of *FLOWERING LOCUS T* in plant development. *Plant, cell & environment*, *35*(10), 1742-1755.

Pnueli, L., Gutfinger, T., Hareven, D., Ben-Naim, O., Ron, N., Adir, N. and Lifschitz, E. (2001). Tomato SP-interacting proteins define a conserved signaling system that regulates shoot architecture and flowering. Plant Cell 13, 2687–2702.

Poethig, R. S. (1990). Phase change and the regulation of shoot morphogenesis in plants. *Science*, *250*(4983), 923.

Powell, L. E. (1987). Hormonal aspects of bud and seed dormancy in temperate-zone woody plants. *HortScience (USA)*.

Prang, L., Stephan, M., Schneider, G. and Bangerth, F. (1997, April). Gibberellin signals originating from apple fruit and their possible involvement in flower induction. In *VIII International Symposium on Plant Bioregulation in Fruit Production 463* (pp. 235-242).

Purohit, A. N. and Tregunna, E. B. (1974). Carbon dioxide compensation and its association with the photoperiodic response of plants. *Canadian journal of botany*, *52*(5), 1146-1148.

Ramirez, H. and Hoad, G. V. (1981, June). Effects of growth substances on fruit-bud initiation in apple. In *Symposium on Growth Regulators in Fruit Production 120* (pp. 131-136).

Reale, L., Sgromo, C., Ederli, L., Pasqualini, S., Orlandi, F., Fornaciari, M., Ferranti, F. and Romano, B. (2009). Morphological and cytological development and starch accumulation in hermaphrodite and staminate flowers of olive (*Olea europaea* L.). *Sexual plant reproduction*, *22*(3), 109-119.

Regnault, T., Davière, J. M., Wild, M., Sakvarelidze-Achard, L., Heintz, D., Bergua, E. C., Diaz, I. L., Gong, F., Hedden, P. and Achard, P. (2015). The gibberellin precursor GA12 acts as a long-distance growth signal in Arabidopsis. *Nature Plants*, *1*(6).

Reig, C., Mesejo, C., Martínez-Fuentes, A. and Agustí, M. (2014). In Loquat (*Eriobotrya japonica* Lindl.) Return Bloom Depends on the Time the Fruit Remains on the Tree. *Journal of Plant Growth Regulation*, *33*(4), 778-787.

Reuther, W., Batchelor, L. D. and Webber, H. J. (1967). The Citrus Industry. Vol.*University of California, Berkeley*.

Roberts, A. V., Blake, P. S., Lewis, R., Taylor, J. M. and Dunstan, D. I. (1999). The effect of gibberellins on flowering in roses. *Journal of plant growth regulation*, *18*(3), 113-119.

Robischon, M., Du, J., Miura, E. and Groover, A. (2011). The Populus class III HD ZIP, popREVOLUTA, influences cambium initiation and patterning of woody stems. *Plant physiology*, *155*(3), 1214-1225.

Rodrigo, M. J., Marcos, J. F., Alférez, F., Mallent, M. D. and Zacarías, L. (2003). Characterization of Pinalate, a novel Citrus sinensis mutant with a fruit-specific alteration that results in yellow pigmentation and decreased ABA content. *Journal of Experimental Botany*, *54*(383), 727-738.

Rodríguez-Falcón, M., Bou, J. and Prat, S. (2006). Seasonal control of tuberization in potato: conserved elements with the flowering response. *Annu. Rev. Plant Biol.*, *57*, 151-180.

Sabatier, S. and Barthélémy, D. (2001). Bud structure in relation to shoot morphology and position on the vegetative annual shoots of *Juglans regia* L.(Juglandaceae). *Annals of Botany*, *87*(1), 117-123.

Sadka, A., Dahan, E., Or, E., and Cohen, L. (2000). NADP+-isocitrate dehydrogenase gene expression and isozyme activity during citrus fruit development. *Plant Science*, *158*(1), 173-181.

Saleh, A., Alvarez-Venegas, R., Yilmaz, M., Le, O., Hou, G., Sadder, M., Al-Abdallat, A., Xia, Y., Lu, G., Ladunga, I. and Avramova, Z. (2008). The highly similar Arabidopsis homologs of trithorax *ATX1* and *ATX2* encode proteins with divergent biochemical functions. *The Plant Cell*, *20*(3), 568-579.

Samach, A. (2013). Congratulations, you have been carefully chosen to represent an important developmental regulator!. *Annals of botany*, *111*(3), 329-333.

Samach, A., Onouchi, H., Gold, S. E., Ditta, G. S., Schwarz-Sommer, Z., Yanofsky, M. F. and Coupland, G. (2000). Distinct roles of *CONSTANS* target genes in reproductive development of Arabidopsis. *Science* 288, 1613–1616.

Schmid, M., Uhlenhaut, N. H., Godard, F., Demar, M., Bressan, R., Weigel, D. and Lohmann, J. U. (2003). Dissection of floral induction pathways using global expression analysis. *Development*, *130*(24), 6001-6012.

Searle, I., He, Y. H., Turck, F., Vincent, C., Fornara, F., Krober, S., Amasino, R. A. and Coupland, G. (2006). The transcription factor *FLC* confers a flowering response to vernalization by repressing meristem competence and systemic signaling in *Arabidopsis*. *Genes Dev*. 20, 898–912.

Searle, N. E. (1965). Physiology of flowering. *Annual Review of Plant Physiology*, *16*(1), 97-118.

Seo, M., Jikumaru, Y. and Kamiya, Y. (2011). Profiling of hormones and related metabolites in seed dormancy and germination studies. In *Seed Dormancy* (pp. 99-111). Humana Press.

Sgamma, T., Jackson, A., Muleo, R., Thomas, B. and Massiah, A. (2014). *TEMPRANILLO* is a regulator of juvenility in plants. *Scientific reports*, *4*.

Shalom, L., Samuels, S., Zur, N., Shlizerman, L., Doron-Faigenboim, A., Blumwald, E. and Sadka, A. (2014). Fruit load induces changes in global gene expression and in abscisic acid (ABA) and indole acetic acid (IAA) homeostasis in citrus buds. *Journal of experimental botany*, *65*(12), 3029-3044.

Shalom, L., Samuels, S., Zur, N., Shlizerman, L., Zemach, H., Weissberg, M., Ophir, R., Blumwald, E. and Sadka, A. (2012). Alternate bearing in citrus: changes in the expression of flowering control genes and in global gene expression in on-versus off-crop trees.

Sheldon, C. C., Jean Finnegan, E., Dennis, E. S. and James Peacock, W. (2006). Quantitative effects of vernalization on *FLC* and *SOC1* expression. *The Plant Journal*, *45*(6), 871-883.

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996). Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Analytical chemistry*, *68*(5), 850-858.

Smidansky, E. D., Meyer, F. D., Blakeslee, B., Weglarz, T. E., Greene, T. W. and Giroux, M. J. (2007). Expression of a modified ADP-glucose pyrophosphorylase large subunit in wheat seeds stimulates photosynthesis and carbon metabolism. *Planta*, *225*(4), 965-976.

Smith, H. and Wareing, P. F. (1964). Gravimorphism in Trees: 2. The Effect of Gravity on Bud-break in Osier Willow. *Annals of Botany*, 283-295.

Smith, J. H. and Benitez, A. (1955). Chlorophylls: analysis in plant materials. In *Modern Methods of Plant Analysis/Moderne Methoden der Pflanzenanalyse* (pp. 142-196). Springer Berlin Heidelberg.

Solomon, M., Belenghi, B., Delledonne, M., Menachem, E. and Levine, A. (1999). The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. *The Plant Cell*, *11*(3), 431-443.

Soltis, D. E., Soltis, P. S., Albert, V. A., Oppenheimer, D. G., dePamphilis, C. W., Ma, H., Frohlich, M. W. and Theißen, G. (2002). Missing links: the genetic architecture of flower and floral diversification. *Trends in Plant Science* 7, 22-31

Southwick, S. M. and Davenport, T. L. (1986). Characterization of water stress and low temperature effects on flower induction in citrus. *Plant Physiology*, *81*(1), 26-29.

Sreekantan, L., Clemens, J., McKenzie, M. J., Lenton, J. R., Croker, S. J. and Jameson, P. E. (2004). Flowering genes in Metrosideros fit a broad herbaceous model encompassing *Arabidopsis* and *Antirrhinum*. *Physiologia Plantarum*, *121*(1), 163-173.

Srinivasan, C. and Mullins, M. G. (1981). Physiology of flowering in the grapevine—a review. *American Journal of Enology and Viticulture*, *32*(1), 47-63.

Stephan, M., Bangerth, F. and Schneider, G. (2001). Transport and metabolism of exogenously applied gibberellins to *Malus domestica* Borkh. cv. Jonagold. *Plant growth regulation*, *33*(2), 77-85.

Strahl, B.D. and Allis, C.D. (2000). The language of covalent histone modifications. Nature 403: 41-45.

Sung, S. and Amasino, R. M. (2004). Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. *Nature*, *427*(6970), 159-164.

Swiezewski, S., Liu, F. Q., Magusin, A. and Dean, C. (2009). Cold-induced silencing by long antisense transcripts of an *Arabidopsis* Polycomb target. *Nature* 462, 799–U122.

Székely, G., Ábrahám, E., Cséplő, Á., Rigó, G., Zsigmond, L., Csiszár, J., Ayaydin, F., Strizhov, N., Jásik, J., Schmelzer, E., Koncz, C. and Szabados, L. (2008). Duplicated P5CS genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis.*The Plant Journal*, *53*(1), 11-28.

Takada, S. and Goto, K. (2003). *TERMINAL FLOWER2*, an *Arabidopsis* homolog of *HETEROCHROMATIN PROTEIN1*, counteracts the activation of *FLOWERING LOCUS T* by *CONSTANS* in the vascular tissues of leaves to regulate flowering time. *The Plant Cell*, *15*(12), 2856-2865.

Takei, K., Sakakibara, H. and Sugiyama, T. (2001). Identification of genes encoding adenylate isopentenyltransferase, a cytokinin biosynthesis enzyme, inArabidopsis thaliana. *Journal of Biological Chemistry*, *276*(28), 26405-26410.

Tamada, Y., Yun, J. Y., chul Woo, S. and Amasino, R. M. (2009). *ARABIDOPSIS TRITHORAX-RELATED7* is required for methylation of lysine 4 of histone H3 and for transcriptional activation of *FLOWERING LOCUS C*. *The plant cell*, *21*(10), 3257-3269.

Tamaki, S., Matsuo, S., Wong, H. L., Yokoi, S. and Shimamoto, K. (2007). Hd3a protein is a mobile flowering signal in rice. *Science*, *316*(5827), 1033-1036.

Tamin, M. (1996). *Involvement of gibberellins in the interaction between vegetative growth and flowering in citrus* (Doctoral dissertation, Ph. D. Thesis, Uni. Jerusalem, Israel).

Tan, F. C. and Swain, S. M. (2007). Functional characterization of *AP3*, *SOC1* and *WUS* homologues from citrus (*Citrus sinensis*). *Physiologia plantarum*, *131*(3), 481-495.

Teper-Bamnolker, P. and Samach, A. (2005). The flowering integrator *FT* regulates *SEPALLATA3* and *FRUITFULL* accumulation in *Arabidopsis* leaves. *The Plant Cell*, *17*(10), 2661-2675.

Tewari, R. K., Kumar, P. and Sharma, P. N. (2005). Signs of oxidative stress in the chlorotic leaves of iron starved plants. *Plant Science*, *169*(6), 1037-1045.

Tisserat, B., Galletta, P. D. and Jones, D. (1990). In vitro flowering from Citrus limon lateral buds. *Journal of plant physiology*, *136*(1), 56-60.

Torti, S., Fornara, F., Vincent, C., Andrés, F., Nordström, K., Göbel, U., Knoll, D., Schoof, H. and Coupland, G. (2012). Analysis of the Arabidopsis shoot meristem transcriptome during floral transition identifies distinct regulatory patterns and a leucine-rich repeat protein that promotes flowering. *The Plant Cell*, *24*(2), 444-462.

Tromp, J. (1982). Flower-bud formation in apple as affected by various gibberellins. *Journal of horticultural science*, 57, 277-282.

Tsukuda, S., Gomi, K., Yamamoto, H., and Akimitsu, K. (2006). Characterization of cDNAs encoding two distinct miraculin-like proteins and stress-related modulation of the corresponding mRNAs in Citrus jambhiri Lush. *Plant molecular biology*, *60*(1), 125-136.

Turck, F., Fornara, F. and Coupland, G. (2008). Regulation and identity of florigen: *FLOWERING LOCUS T* moves center stage. *Annu. Rev. Plant Biol.*, *59*, 573-594.

Valiente, J. I. and Albrigo, L. G. (2004). Flower bud induction of sweet orange trees [Citrus sinensis (L.) Osbeck]: effect of low temperatures, crop load, and bud age. *Journal of the American Society for Horticultural Science*, *129*(2), 158-164.

Verreynne, J. S. and Lovatt, C. J. (2009). The effect of crop load on budbreak influences return bloom in alternate bearing 'Pixie' mandarin. *Journal of the American Society for Horticultural Science*, *134*(3), 299-307.

Wagner, D., Sablowski, R. W. and Meyerowitz, E. M. (1999). Transcriptional activation of *APETALA1* by *LEAFY*. *Science*, *285*(5427), 582-584.

Walton, E. F., Fowke, P. J., Weis, K. and McLeay, P. L. (1997). Shoot axillary bud morphogenesis in kiwifruit (*Actinidia deliciosa*). *Annals of Botany*, *80*(1), 13-21.

Wang, R., Farrona, S., Vincent, C., Joecker, A., Schoof, H., Turck, F., Alonso-Blanco, C., Couplang, G. and Albani, M. C. (2009). *PEP1* regulates perennial flowering in *Arabis alpina. Nature*, *459*(7245), 423-427.

Wang, X., Zhang, Y., Ma, Q., Zhang, Z., Xue, Y., Bao, S. and Chong, K. (2007). *SKB1*-mediated symmetric dimethylation of histone H4R3 controls flowering time in Arabidopsis. *The EMBO journal*, *26*(7), 1934-1941.

Wang, Y., Li, L., Ye, T., Lu, Y., Chen, X. and Wu, Y. (2013). The inhibitory effect of ABA on floral transition is mediated by ABI5 in Arabidopsis. *Journal of experimental botany*, *64*(2), 675-684.

Wang, Y., Liu, C., Yang, D., Yu, H. and Liou, Y. C. (2010). Pin1At encoding a peptidyl-prolyl cis/trans isomerase regulates flowering time in Arabidopsis.*Molecular cell*, *37*(1), 112-122.

Wareing, P. F. (1959). Problems of juvenility and flowering in trees. *Journal of the Linnean Society of London, Botany*, *56*(366), 282-289.

Weigel, D. and Nilsson, O. (1995). A developmental switch sufficient for flower initiation in diverse plants. *Nature*, *377*(6549), 495-500.

Wellensiek, S. J. (1970). Floral hormones in *Silene armeria* L. and *Xanthium strumarium* L. *Zeitschrift fur Pflanzenphysiologie*.

Werner, T., Motyka, V., Laucou, V., Smets, R., Van Onckelen, H. and Schmülling, T. (2003). Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *The Plant Cell*, *15*(11), 2532-2550.

Wigge, P. A., Kim, M. C., Jaeger, K. E., Busch, W., Schmid, M., Lohmann, J. U. and Weigel, D. (2005). Integration of spatial and temporal information during floral induction in *Arabidopsis*. *Science*, *309*(5737), 1056-1059.

Wilkie, J. D., Sedgley, M. and Olesen, T. (2008). Regulation of floral initiation in horticultural trees. *Journal of Experimental Botany*, *59*(12), 3215-3228.

Wolf, S., Hématy, K., and Höfte, H. (2012). Growth control and cell wall signaling in plants. *Annual review of plant biology*, *63*, 381-407.

Yahata, D., Matsumoto, K. and Ushijima K. (2006). The effect of the time of fruit harvest on flower formation and carbohydrate contents in shoots of Wase Satsuma trees, *J. Jpn. Soc. Hortic. Sci*. 75.

Yamaguchi, A., Wu, M. F., Yang, L., Wu, G., Poethig, R. S. and Wagner, D. (2009). The microRNA-regulated SBP-Box transcription factor *SPL3* is a direct upstream activator of *LEAFY*, *FRUITFULL*, and *APETALA1*. *Developmental cell*, *17*(2), 268-278.

Yamaguchi, N., Winter, C. M., Wu, M. F., Kanno, Y., Yamaguchi, A., Seo, M. and Wagner, D. (2014). Gibberellin acts positively then negatively to control onset of flower formation in *Arabidopsis*. *Science*, *344*(6184), 638-641.

Zabaleta, E., Oropeza, A., Assad, N., Mandel, A., Salerno, G. and Herrera-Estrella, L. (1994). Antisense expression of chaperonin 60β in transgenic tobacco plants leads to abnormal phenotypes and altered distribution of photoassimilates. *The Plant Journal*, *6*(3), 425-432.

Zambryski, P. (2008). Plasmodesmata. *Current Biology*, *18*(8), R324-R325.

Zeevaart, J. A. (1976). Physiology of flower formation. *Annual Review of Plant Physiology*, *27*(1), 321-348.

Zemach, A. and Grafi, G. (2007). Methyl-CpG-binding domain proteins in plants: interpreters of DNA methylation. *Trends in plant science*, *12*(2), 80-85.

Zhang, X., Yazaki, J., Sundaresan, A., Cokus, S., Chan, S. W. L., Chen, H., Henderson, I. R., Shinn, P., Pellegrini, M., Jacobsen, S. E. and Ecker, J. R. (2006). Genome-wide high-resolution mapping and functional analysis of DNA methylation in Arabidopsis. *Cell*, *126*(6), 1189-1201.

Zilberman, D., Gehring, M., Tran, R. K., Ballinger, T. and Henikoff, S. (2007). Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription. *Nature genetics*, *39*(1), 61-69.

Ziv, D., Zviran, T., Zezak, O., Samach, A. and Irihimovitch, V. (2014). Expression Profiling of *FLOWERING LOCUS T*-Like Gene in Alternate Bearing 'Hass' Avocado Trees Suggests a Role for *PaFT* in Avocado Flower Induction. *PlosOne*